University of Alberta

Alternative Oxidase Regulatory Mutants in *Neurospora crassa*

by

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Abstract

Mitochondria are the major sites of ATP generation in eukaryotic cells. While most mitochondrial proteins are encoded by nuclear genes, a few proteins necessary for oxidative phosphorylation are encoded by the mitochondrial genome. Since nuclear gene expression is modulated in response to mitochondrial conditions, an as yet unknown signaling system between mitochondria and the nucleus must exist to allow the coordinate gene expression necessary for proper mitochondrial function. The nuclear gene *aod-1*, which encodes the alternative oxidase in *Neurospora crassa*, provides a system to study this signaling. Alternative oxidase shows specific induction: it is produced when the primary oxidative phosphorylation pathway is inhibited, but is absent when the oxidative pathway is not compromised.

Mutants in this unknown signaling pathway were identified through an EMS mutagenesis screen of a strain containing a reporter construct of the *aod-1* promoter fused to the tyrosinase structural gene. Fifteen putative regulatory mutants comprising five novel complementation groups were identified in the screen. All five mutant loci fail to induce alternative oxidase under inducing conditions. Three of the mutants produce no *aod-1* mRNA or protein under inducing conditions, while the other two mutants produce some protein under inducing conditions. None of the mutations affected global mitochondrial function or growth of the strains, suggesting that the mutations affected *aod-1* regulation specifically

The gene affected in the *aod-5* mutant was cloned and found to encode a Zn(II)2Cys6 fungal transcription factor. This protein is predicted to regulate transcription by binding DNA as a dimer. It also has a PAS domain which may be involved in signal

transduction.

The assembly of AOD1 protein in *N. crassa* was also examined. AOD1 is an interfacial integral membrane protein associated with the mitochondrial inner membrane. This protein is specifically localized to mitochondria within the cell. It can be partially extracted from the membrane with sodium carbonate (pH 11.5) treatment and is also released from the membrane upon sonication.

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List of Abbreviations

2D	two dimensional
3D	three dimensional
A. maculatum	*Arum maculatum*
A. fumigatus	*Aspergillus fumigatus*
A. nidulans	*Aspergillus nidulans*
A. niger	*Aspergillus niger*
A. thaliana	*Arabidopsis thaliana*
A	adenine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
Ant A	antimycin A
ATP	adenosine triphosphate
Ben	benomyl
bHLH/Zip	basic helix-loop-helix zipper
BLAST	basic local alignment search tool
bp	base pair
°C	degree Celsius
C	cytosine
C. albicans	*Candida albicans*
cAMP	cyclic adenosine monophosphate
CAP	chloramphenicol
cDNA	complimentary deoxyribonucleic acid
CORR	co-localization for redox regulation
Cox	cytochrome oxidase
CRE	cyclic adenosine monophosphate response element
cys	cysteine
Δ	deletion
D. melanogaster	*Drosophila melanogaster*
Da	Dalton
DEPC	diethylpyrocarbonate
DHFR	dihydrofolate reductase
DMQ Hydroxylase	demethoxyquinone hydroxylase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DR	dioxin receptor
E. coli	*Escherichia coli*
ECL	enzyme catalyzed light generation
EMS	ethyl methanesulfonate
EMSA	electrophoretic mobility shift assay
ETC	electron transport chain
EPR	electroparamagnetic resonance
F1β	F1beta subunit of the ATP synthase

FADH2	flavin adenine dinucleotide, reduced form
G	guanine
G. max	*Glycine max*
gDNA	genomic DNA
GIP	general import pore
glu	glutamic acid
GTP	guanosine triphosphate
h	hour
H. capsulatum	*Histoplasma capsulatum*
his	histidine
HRP	horse radish peroxidase
Hsp	heat shock protein
IMS	intermembrane space
IPTG	isopropyl-beta-D-thiogalactopyranoside
kbp	kilo base pair
KCN	potassium cyanide
kDa	kilo Dalton
kV	kilo volt
L	litre
LB	Luria-Bertani
LG	linkage group
LRE	light response element
μg	microgram
μl	microlitre
μm	micrometer
M	molar
M. grisea	*Magnaporthe grisea*
MCS	multiple cloning site
mg	milligrams
MIM	mitochondrial inner membrane
min	minute
ml	milliliter
mM	millimolar
MOM	mitochondrial outer membrane
MOPS	4-morpholinepropanesulfonic acid
MHR	middle homology region
mRNA	messenger ribonucleic acid
ms	millisecond
mtDNA	mitochondrial DNA
N. crassa	*Neurospora crassa*
N. tabacum	*Nicotiana tabacum*
NADH	nicotinamide adenine dinucleotide, reduced form
NiNTA	nickel-nitrilotriacetic acid
NLS	nuclear localization signal
Ω	ohms
OD	optical density

ORF	open reading frame
P. anserina	*Podospora anserina*
PAGE	polyacrylamide gel electrophoresis
PAS	Period Arnt Single-Minded
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PK	proteinase K
PMSF	phenylmethylsulfonyl fluoride
RIP	repeat induced point mutation
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase PCR
RTG	retrograde response
s	second
S. cerevisiae	*Saccharomyces cerevisiae*
S. gutattum	*Sauromatum guttatum*
S. pombe	*Schizosaccharomyces pombe*
SAM	sorting and assembly machinery
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHAM	salicylhydroxamic acid
T	thymine
T. brucei	*Trypanosoma brucei*
TCA	trichloroacetic acid
TIM	translocase of the inner mitochondrial membrane
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer RNA
TOB	topogenesis of mitochondrial outer membrane beta barrel proteins
TOM	translocase of the outer mitochondrial membrane
UTR	untranslated region
WCC	white collar complex

1. Introduction

1.1 Mitochondria

The mitochondrion is found in nearly all eukaryotic cells. This organelle plays a key role in numerous cellular processes, including ATP generation, amino acid metabolism, fatty acid oxidation, and iron-sulphur cluster synthesis. While a few of the important proteins of the oxidative phosphorylation system are encoded by genes on mitochondrial DNA (mtDNA), the vast majority of mitochondrial proteins are nuclear gene products. Coordinated expression from both genomes is thus necessary for proper mitochondrial function (JOSEPH-HORNE *et al.* 2001; VANLERBERGHE and MCINTOSH 1997).

1.2 Mitochondrial Structure

Mitochondria are double-membrane bound organelles and as such they consist of four compartments: the mitochondrial outer membrane (MOM), the intermembrane space (IMS), the mitochondrial inner membrane (MIM), and the matrix. The inner membrane is highly folded into structures known as cristae. This infolding increases the surface area of the MIM available for oxidative phosphorylation. The structure of the MIM is highly dynamic and is capable of adjusting to different cellular conditions (VAN DER GIEZEN and TOVAR 2005). Electron tomography has revealed that cristae are not simple invaginations of the MIM, but have a structure and protein content that is different than that of the peripheral inner membrane (that portion of the membrane that roughly parallels the MOM) (MANNELLA 2006). Cristae are connected to the peripheral MIM by short tubular regions, and this organization is thought to limit diffusion of IMS and MIM proteins in and out of cristae. The resulting bottleneck effect may have functional implications for the mitochondria. Some electron transport system proteins in the MIM are up to twice as abundant in cristae as compared to the peripheral MIM. Computer models have even predicted that limited diffusion may allow localized depletion or elevation of ATP and ADP concentrations to occur in individual cristae (MANNELLA 2006). The structure of cristae is highly flexible, and ranges from large compartments to a highly interconnected lattice in the matrix. Cristae may have many or few connections with the peripheral MIM, and are thought to be able to divide and fuse with each other (MANNELLA *et al.* 2001).

Mitochondria themselves are capable of dividing and fusing, independent of the cell cycle. Rather than existing as numerous, discrete structures within the cell, mitochondria are seen to form a highly dynamic reticular network. This network is maintained through a balance between constant mitochondrial fusion and fission events (MEEUSEN *et al.* 2004; SHAW and NUNNARI 2002). An excess of fission leads to highly fragmented mitochondria, while an excess of fusion results in fewer, larger compartments (YAFFE 1999). Fission and fusion of mitochondria are unlike that of other organelles in the eukaryotic cell. Unlike endoplasmic reticulum, for example, mitochondria must successfully fuse and divide two distinct membranes (MEEUSEN and NUNNARI 2005).

1.3 Mitochondria Origin

Mitochondria are thought to have evolved from an α-proteobacterium that was engulfed by an early eukaryotic or proto-eukarytoic cell to form an endosymbiotic relationship approximately 1.5 billion years ago (GRAY 1999). The driving force behind this endosymbiosis is not entirely clear, but two hypotheses are currently favoured. The first is that the metabolism of the endosymbiont was able to detoxify oxygen, which was becoming increasingly common in the environment (ANDERSSON *et al.* 2003). The second hypothesis is the hydrogen hypothesis, which supposes that the eubacterial endosymbiont provided hydrogen and carbon dioxide as waste products and that the hydrogen was used by a methanogenic archaeon host as an energy source (MARTIN and MULLER 1998).

Eukaryotic species that lack mitochondria were originally thought to represent ancient lineages that branched before endosymbiosis, however these species appear to carry degenerate mitochondria, and also have genes that appear to be of mitochondrial origin in their nuclear DNA (EMBLEY *et al.* 2003).

Hydrogenosomes are energy-producing double-membrane bound organelles that generate ATP through fermentation, and produce hydrogen as a byproduct (MARTIN 2005; MARTIN *et al.* 2001). Some hydrogenosomes even contain their own genome, a genome which closely resembles mitochondrial genomes of related mitochondria-containing species (BOXMA *et al.* 2005). Mitosomes are also organelles with double membranes, but they do not produce ATP and they lack a genome (LEON-AVILA and TOVAR 2004). Both structurally similar organelles could share a common ancestor with mitochondria.

Interestingly, both mitosomes from *Giardia intestinalis* (TOVAR *et al.* 2003) and hydrogenosomes in *Trychomonas vaginalis* (SUTAK *et al.* 2004) contain conserved proteins involved in iron-sulphur cluster synthesis, an essential process that occurs in mitochondria (DOLEZAL *et al.* 2005). Further, proteins targeted to one organelle are imported into the other organelle *in vivo*. Sequence analysis has also discovered a homologue of a mitochondrial import machinery component in both these species, and in each case this protein is targeted to the organelle (DOLEZAL *et al.* 2005). This observation further supports the theory that mitosomes and hydrogenosomes share a common ancestor with mitochondria.

1.4 Mitochondrial Functions

1.4.1 Metabolism

Mitochondria play an important role in many cellular metabolic processes. They are involved in amino acid synthesis, ketone body synthesis, the urea cycle, as well as glycolysis and the Krebs cycle, which are important sources of carbon skeletons. Mitochondria also store calcium and are involved in maintaining calcium homeostasis in the cell.

1.4.2 Oxidative Phosphorylation

One of the most important functions of mitochondria is the production of energy; mitochondria generate the bulk of cellular ATP through the process of oxidative phosphorylation. In this process electrons pass through a series of protein complexes called the electron transport chain (ETC) (Figure 1). Electrons are donated to the ETC by donor molecules generated in the Krebs cycle, glycolysis or β-oxidation of fatty acids

(BARTLETT and EATON 2004). The energy of electrons passing through the chain is harnessed by Complexes I, III and IV of the ETC to pump protons across the MIM, thus generating a proton gradient (SARASTE 1999). At the end of the chain, electrons are donated to oxygen molecules to form water. Protons are able to flow back across the MIM through the ATPase, also known as Complex V. This protein complex uses the energy of the proton gradient to phosphorylate ADP to ATP. *Saccharomyces cerevisiae*, unlike many other eukaryotes, lacks Complex I (JOSEPH-HORNE *et al.* 2001). Several chemicals can interrupt electron flow. Antimycin A and cyanide inhibit Complexes III and IV respectively, while chloramphenicol inhibits mitochondrial translation, and therefore production of mitochondrially encoded components of the ETC (JOSEPH-HORNE *et al.* 2001).

1.4.3 Iron-Sulphur cluster synthesis

Iron-sulphur clusters are cofactors for many proteins involved in a variety of processes, including cellular respiration (JOSEPH-HORNE *et al.* 2001). Proteins containing iron-sulphur clusters are found in different compartments of the eukaryotic cell including the nucleus, mitochondria and the cytosol (LILL and MUHLENHOFF 2005). Although there are proteins involved in iron-sulphur cluster maturation found in each of these compartments, mitochondria play an essential role in the assembly of all cellular iron-sulphur clusters. The primary assembly machinery consists of approximately ten proteins found in mitochondria (LILL and MUHLENHOFF 2005). These proteins are highly similar to bacterial iron-sulphur cluster assembly factors, and the eukaryotic assembly factors are thought to have been derived from the endosymbiotic ancestor of mitochondria (LILL and

KISPAL 2000). In yeast, deletion of these assembly proteins is lethal or results in a severe growth defect, even on rich media (LILL and KISPAL 2000).

Iron-sulphur clusters are important in mitochondria because enzymes containing iron-sulphur clusters synthesize the heme groups necessary in the function of the cytochromes of the ETC. Many assembly factors of iron-sulphur clusters themselves contain iron-sulphur clusters and are thus important for their own assembly (LILL and MUHLENHOFF 2005). Because yeast can grow by fermentation, and can therefore dispense with the ETC, the use of iron-sulphur clusters in the ETC does not alone explain the essential nature of the mitochondrial iron-sulphur assembly machinery. It has now been discovered that the iron-sulphur cluster-containing protein Rli1p is required for ribosome biogenesis (KISPAL *et al.* 2005; YARUNIN *et al.* 2005). This highly conserved protein may process rRNAs, and this function explains the lethality of deletions of mitochondrial iron-sulphur cluster assembly factors. This is the first instance of an essential function of mitochondria in yeast.

Defects in assembly of iron-sulphur clusters have also been implicated in human disease. The human mitochondrial protein frataxin is thought to be required for insertion of iron into iron-sulphur clusters. Mutations in this gene lead to the disease Friedreich Ataxia, which is characterized by elevated mitochondrial iron concentrations, lower production of heme groups, and consequently decreased levels of the cytochromes of the ETC (PUCCIO and KOENIG 2002).

1.5 Mitochondrial DNA

All mitochondria contain their own DNA, found in the matrix of the organelle. The protein-coding genes of this genome are translated on mitochondrial ribosomes and function in mitochondria. The largest gene complement in mtDNA is found in the 69 kbp mitochondrial genome of the protist *Reclinomonas americana*, which in addition to tRNAs and rRNAs necessary for mitochondrial translation, contains 67 protein-coding genes (LANG *et al.* 1997). The parasite *Plasmodium falciparum* offers the example of the smallest mtDNA coding capacity, carrying only three protein-coding genes (GRAY 1999). In comparison, humans have a 16.5 kbp mtDNA with 13 protein-coding genes (SHADEL and CLAYTON 1997) while the *Neurospora crassa* mtDNA is 64.8 kbp and encodes 15 proteins (KENNELL *et al.* 2004). Since the original endosymbiont had its own complete genome, the reduced genomes seen in mitochondria are indicative of the fact that the nucleus now houses most genes for maintaining mitochondrial function. The different complement of mitochondrial genes observed in different organisms suggests that endosymbiont genes were not transferred *en masse*, but that different genes were transferred to the nucleus throughout evolution; in fact gene transfer from mitochondria is continuing in flowering plants (ADAMS *et al.* 2000).

It has been shown experimentally that plasmids transformed into mitochondria can readily move to the nucleus where the genes they carry can function (THORSNESS and FOX 1990). Hypotheses have been presented as to why gene transfer to the nucleus might be preferred. Since mitochondria lack DNA repair machinery, and are major producers of damaging reactive oxygen species (ROS), transfer of a gene to the nucleus might limit its exposure to damage, while also providing a way of repairing that damage. Also, because the mitochondrial genome replicates asexually, there is no opportunity to repair mutations by recombination. In contrast, the nuclear genome undergoes sexual replication, where transferred mitochondrial genes can benefit from this repair mechanism (BLANCHARD and LYNCH 2000).

In spite of potential advantages to transferring to the nucleus, some genes remain in all mitochondrial genomes, a situation for which there are two main hypotheses. The first is the hydrophobicity hypothesis (VON HEIJNE 1986b). The proteins encoded by mtDNA are components of the ETC. These are highly hydrophobic integral membrane proteins and it is thought that their highly hydrophobic nature prevents them from being translated on cytosolic ribosomes and imported into mitochondria. By being translated in the matrix they can be directly inserted into the MIM where they are functional (VON HEIJNE 1986b). One example of the effect of protein hydrophobicity on gene transfer is the Cox2 gene in plants. This gene has been transferred to the nucleus only in some members of the legume family, and a mitochondrial copy remains. The nuclear copy of Cox2 has changed so that the protein it encodes is less hydrophobic than that encoded by the mitochondrial gene (DALEY *et al.* 2002). This demonstrates the importance of hydrophobicity in proper localization and import of mitochondrial proteins encoded by nuclear genes.

The second hypothesis is the CORR hypothesis: colocalization for redox regulation (ALLEN 2003). This hypothesis states that mitochondrial gene expression is regulated directly by conditions in the matrix, specifically the redox state reflecting the activity of the ETC and the Krebs cycle. Genes that have not transferred to the nucleus have not done so because their regulation depends directly on mitochondrial conditions (Allen 2003).

1.6 Mitochondrial Protein Import

Most mitochondrial proteins are encoded by nuclear genes and translated on cytosolic ribosomes and because of this they must be imported and sorted into the correct mitochondrial compartment to be functional. Many nuclear-encoded mitochondrial genes contain an N-terminal 20-80 amino acid targeting sequence that directs them to the mitochondrion. The amino acids of the targeting sequences are usually rich in positively charged and hydroxylated residues and they form an amphipathic helix (von Heijne 1986a). Other proteins contain internal targeting sequences.

Proteins reaching the mitochondrion are imported by the general import pore or TOM complex (translocase of the outer membrane), which has several major components. The pore itself is formed by the essential β-barrel protein Tom40 (Ahting *et al.* 2001; Hill *et al.* 1998). Tom20, Tom22 and Tom70 are receptors which interact with proteins targeted to mitochondria (Hoppins *et al.* 2004).

After crossing the outer membrane, proteins may reside in the IMS or they can be sorted into one of the other compartments. The TOB/SAM complex (topogenesis of mitochondrial outer-membrane beta-barrel proteins/sorting and assembly machinery) inserts β-barrel proteins into the MOM while TIM complexes (translocase of the inner membrane) direct proteins to the MIM or to the matrix. Proteins without an N-terminal targeting sequence interact with the TIM22 complex and are inserted into the MIM (Hoppins *et al.* 2004). A large class of multitopic membrane-spanning proteins with N-terminal targeting sequences interact with the TIM23 complex and are inserted into the MIM or translocated into the matrix (HOPPINS *et al.* 2004).

Some proteins are inserted into the MIM from the matrix. Some of these proteins are encoded by nuclear genes, translocated from the cytosol to the matrix, and then exported back into the MIM. Other proteins are encoded by mtDNA, translated on mitochondrial ribosomes and subsequently inserted into the MIM. This is achieved by the interaction of these proteins with the OXA1 protein (oxidase assembly), a highly conserved protein which is homologous to YidC in bacteria (SCOTTI *et al.* 2000). OXA1 is an essential protein in *N. crassa* and a deficiency of OXA1 leads to deficiencies in complexes I and IV of the ETC (NARGANG *et al.* 2002).

1.7 Mitochondria and Human Disease

1.7.1 Dysfunction

Mitochondria are often involved in human disease. Mitochondrial dysfunction can arise from mutations of mtDNA or nuclear genes, and thus both genomes are implicated in disease (DIMAURO 2004). Because mitochondria produce the bulk of a cell's ATP, the effects of mitochondrial dysfunction appear most readily in those tissues that require large amounts of energy, namely muscles, neurons and the liver. Since mitochondria are also involved in apoptosis, defects in programmed cell death that lead to disease may also involve mitochondria. In addition to metabolic disorders arising from oxidative phosphorylation or amino acid metabolism defects in mitochondria, the organelle has also been implicated in a variety of neurodegenerative disorders including Parkinson's Disease and Alzheimer's Disease (MANFREDI and BEAL 2000).

1.7.2 Aging

Mitochondria have been implicated in aging because changes in mitochondria have been correlated with age and with the effects of aging. Aged cells produce less ATP and have less efficient oxidative phosphorylation than young cells (OZAWA 1999). The number of mtDNA mutations also increases with age (CORRAL-DEBRINSKI *et al.* 1992; CORTOPASSI and WONG 1999), leading to reduced mitochondrial function. The free radical theory of aging (HARMAN 1956) suggests that ROS cause oxidative damage to mtDNA and the ETC, and that this damage in turn leads to mitochondrial dysfunction and increased production of ROS. In support of this theory are correlations that show caloric restriction decreases ROS production and increases lifespan (BARJA 2004). Mitochondrial damage accumulated over a lifetime could eventually impair mitochondrial function to the point where apoptosis is triggered leading to tissue degeneration and other affects of aging (OZAWA 1997; OZAWA 1999).

Mice have provided a useful model for studying aging in mammals. Like humans, they accumulate mtDNA mutations with age (KHAIDAKOV *et al.* 2003) and in addition they have also provided experimental evidence relating mtDNA mutations to aging, rather than just correlation studies. A proofreading deficient mtDNA polymerase creates three to five times the normal number of mtDNA mutations, and mice carrying this mutant polymerase had decreased lifespan and showed premature signs of aging (TRIFUNOVIC *et al.* 2004). These mutations decreased ATP production, and showed a causative link between mtDNA mutations and aging. Interestingly, there was no induction of ROS protection machinery in these mice, suggesting that no there was no increase in ROS production corresponding to accumulated mtDNA mutations (TRIFUNOVIC *et al.* 2004). Further investigation of ROS showed that there was no significant difference in ROS levels between wild-type and polymerase mutant mice, even though cellular respiration was severely impaired (TRIFUNOVIC *et al.* 2005). Although there was a small increase in the amount of ROS damage, this was not enough to trigger ROS defense mechanisms (TRIFUNOVIC *et al.* 2005). Another experiment in mice showed that overexpression of human catalase in the mitochondria of transgenic mice also influenced aging. These mice showed increased lifespan, lower levels of reactive oxygen damage and decreased levels of hydrogen peroxide production (SCHRINER *et al.* 2005). The conclusions of these two experiments are not necessarily mutually exclusive, since the mutations created by the error-prone polymerase may in fact reduce ROS generation by impairing overall mitochondrial function, and an error-prone polymerase could itself be generated *in vivo* by ROS damage (LOEB *et al.* 2005). Clearly more work will be required to determine the role of mitochondria in aging.

1.8 Coordinate Genome Regulation – The RTG system

S. cerevisiae possesses a retrograde response (RTG) system whereby nuclear genes are regulated by mitochondrial conditions. In the RTG system three genes, *RTG1, RTG2* and *RTG3* are required for the induction of the peroxisomal isoform of citrate synthase *CIT2*. *CIT2* is expressed as a compensatory measure when mitochondrial function is compromised, such as when mtDNA is lost (LIAO and BUTOW 1993). *RTG1* and *RTG3* encode basic helix–loop-helix-leucine zipper (bHLH-zip) transcription factors which bind the *CIT2* promoter as a heterodimer (JIA *et al.* 1997; LIAO and BUTOW 1993). While both proteins are necessary for DNA binding, only Rtg3p contains an activation domain (ROTHERMEL *et al.* 1997).

The activity of these transcription factors is regulated in part by their cellular localization. Under conditions where *CIT2* expression is low, Rtg1p and Rtg3p form a highly phosphorylated complex localized to the cytosol. Under conditions of high *CIT2* expression, such as when mtDNA is lost, the Rtg1p/Rtg3p complex is dephosphorylated and each protein is translocated to the nucleus. In the nucleus they form a heterodimer and activate *CIT2* transcription (SEKITO *et al.* 2000). The role of Rtg2p is unclear, but it has a cytoplasmic localization and is necessary for Rtg1p and Rtg3p translocation.

Although there is an *N. crassa* homologue of *RTG2*, there do not appear to be homologues of *RTG1* or *RTG3* present (M. Chae, personal communication). This does not necessarily preclude the existence of a homologous pathway in *N. crassa* since *rtg-2* could activate different transcription factors which perform the same functions as Rtg1p and Rtg3p.

1.9 Alternative Oxidase

The nuclear gene *aod-1*, which encodes the mitochondrially located alternative oxidase in *N. crassa*, provides a system to study the signaling needed to coordinate nuclear and mitochondrial gene regulation. This gene is specifically induced in response to perturbations in mitochondrial function (BERTRAND *et al.* 1983), and therefore a signaling pathway between mitochondria and the nucleus must exist to carry out this induction.

Alternative oxidase is a second terminal oxidase found associated with the matrix side of the MIM (Figure 1). It transfers electrons directly from ubiquinone in the ETC to reduce molecular oxygen to water, bypassing proton pumping sites at Complexes III and IV. Respiration *via* alternative oxidase can be distinguished from that of the cytochrome mediated ETC since it is insensitive to cyanide, but is sensitive to other inhibitors such as salicylhydroxamic acid (SHAM) and propyl gallate (VANLERBERGHE and MCINTOSH 1997).

1.10 Alternative Oxidase in Evolution

Alternative oxidase is found extensively in higher plants (CONSIDINE *et al.* 2002) and fungi (JOSEPH-HORNE *et al.* 2001), as well as the alga *Chlamydomonas reinhardtii* (BAURAIN *et al.* 2003), and parasitic protists such as *Trypanosoma brucei* (AJAYI *et al.* 2002). Recent genome sequencing projects have increased the range of organisms containing this mitochondrial protein, for the first time identifying alternative oxidase genes in some animal species (MCDONALD and VANLERBERGHE 2004). Alternative oxidase homologues were found in mollusk, nematode, diatom, red algae and primitive chordate species. The function of alternative oxidase in these species is unknown, but in the marine species it may provide a mechanism to deal with variable environmental oxygen levels or high sulphide concentrations which can inhibit cytochrome oxidase (MCDONALD and VANLERBERGHE 2004).

A prokaryotic evolutionary origin for alternative oxidase has been suggested by the identification of a protein in the α-proteobacterium *Novosphingobium aromaticivorans*, (FINNEGAN *et al.* 2003; STENMARK and NORDLUND 2003). This protein bears 58% amino acid identity to alternative oxidase of *Arabidopsis thaliana*, and like other plant or fungal alternative oxidase is able to restore respiration to heme-deficient *Escherichia coli* (STENMARK and NORDLUND 2003). Mass sequencing of prokaryotic genomic DNA collected in the Sargasso Sea identified 67 putative prokaryotic alternative oxidases. These enzymes showed conservation of important residues common with eukaryotic alternative oxidases. Phylogenic analysis of bacterial alternative oxidase proteins suggests that the eukaryotic proteins may have originated from the original endosymbiont that gave rise to mitochondria (MCDONALD *et al.* 2003).

1.11 Alternative Oxidase Structure

Alternative oxidase is an interfacial, integral membrane protein that has regions embedded in the MIM, but lacks a transmembrane domain (BERTHOLD and STENMARK 2003). The active site is a non-heme diiron binding motif which has been confirmed by a lack of activity in iron-starved cells of *Histoplasma anomala* (BERTHOLD *et al.* 2000) and *T. brucei* (CHAUDHURI *et al.* 2002) and also by EPR study (BERTHOLD *et al.* 2002). The active site is thought to consist of four short alpha-helices containing two conserved Glu-X-X-His motifs that form a binuclear iron centre. No crystal structure has yet been solved for alternative oxidase, but several models for its general structure have been suggested. The latest model (ANDERSSON and NORDLUND 1999) agrees most closely with structural predictions for other membrane-bound diiron carboxylate enzymes

(BERTHOLD and STENMARK 2003), and is supported by site directed mutagenesis experiments where mutations in the putative active site eliminate activity (AFFOURTIT *et al.* 2002; AJAYI *et al.* 2002; ALBURY *et al.* 2002; CHAUDHURI *et al.* 2002; UMBACH *et al.* 2002). Alternative oxidase does not appear to be part of a respiratory supercomplex comprising other ETC complexes in plants (EUBEL *et al.* 2003; KRAUSE *et al.* 2004a) or fungus (KRAUSE *et al.* 2004b).

1.12 Plant Alternative Oxidase

Plant alternative oxidase enzymes are often encoded by a small multigene family whose members show differential expression. *Glycine max*, for example, has three genes. *Aox1* is specifically induced by antimycin A, citrate, salicylic acid and growth at low temperatures (10°C) while *aox2* expression is not observed under any of these conditions. *Aox3* is observed in uninduced cells (DJAJANEGARA *et al.* 2002). *Zea mays* also has three genes: *aox1* is observed only in tassels while *aox2* and *aox3* are expressed in all tissues, and are highly inducible by antimycin A and cyanide (KARPOVA *et al.* 2002). Generally, plant alternative oxidase genes fall into one of two classes. Aox Type 1 genes are expressed in most tissues, but only when induced by external factors such as ETC inhibitors, pathogen attack or other stresses, and are found in all plants examined. Aox Type 2 genes appear to have a housekeeping role and are constitutively expressed at low levels, often in a tissue specific manner or at certain stages of development (CONSIDINE *et al.* 2002). Aox Type 2 genes are found in eudicots, but not monocots (JUSZCZUK and RYCHTER 2003). *A. thaliana* has the typical Aox Type 1 (*Aox1*) stress inducible and Aox Type 2 (*Aox2*) housekeeping genes, but *Aox2* is also induced under specific stress conditions (CLIFTON *et al.* 2005). When chloroplast function is disrupted Aox2 expression increases, suggesting some inter-organellar cross talk. The mechanism of this induction is not yet known (CLIFTON *et al.* 2005).

Plant alternative oxidase can be induced by a variety of internal and environmental stresses. Pathogen attack (MAXWELL *et al.* 1999; ORDOG *et al.* 2002; SIMONS *et al.* 1999), cold (JUSZCZUK and RYCHTER 2003), phosphate deficiency (JUSZCZUK *et al.* 2001; PARSONS *et al.* 1999), salt (OTTOW *et al.* 2005) and drought (RIBAS-CARBO *et al.* 2005) all increase alternative oxidase activity. It could be that these plant stresses affect mitochondrial function and therefore induce alternative oxidase, or that alternative oxidase is part of a general stress response. In *A. thaliana* a 93 bp region in the promoter of *AtAOX1a* is required for the strong induction of that gene (DOJCINOVIC *et al.* 2005). The presence of numerous transcription factor binding sites in the region means that the precise mechanism by which transcription of this gene is induced will require further study to be understood.

In addition to transcriptional regulation, several mechanisms exist in plants for regulating the activity of the alternative oxidase protein. Plant alternative oxidase is thought to function as a homodimer. A long N-terminal tail contains a conserved cysteine, called Cys_I, postulated to form disulphide bridges (VANLERBERGHE and MCINTOSH 1997). Experiments *in vitro* showed that di-sulphide bridge reduction increased enzyme activity (AFFOURTIT *et al.* 2001), however experiments using *Nicotiana tabacum* mitochondria isolated in the presence of pyruvate (VANLERBERGHE *et al.* 1999) or *Poa annua* whole root cells (MILLENAAR *et al.* 2000), showed that most of the protein is expected to be in the reduced form *in vivo*. Disulphide bridges may therefore not be the most important means of regulating enzyme activity.

Enzyme activity can also be modulated by α-keto acids, such as pyruvate. These molecules bind the conserved $\mathrm{Cys_I}$ to stimulate enzyme activity, possibly through interactions with the cysteine's sulphydryl groups to form a thiohemiacetal group (JUSZCZUK and RYCHTER 2003; RHOADS *et al.* 1998). Since high pyruvate levels slow metabolism and electron transport, even when there is high demand for carbon skeletons, increased alternative oxidase activity may help restore metabolic balance (AFFOURTIT *et al.* 2001). Amino acid substitutions at this cysteine dramatically alter stimulation. Replacing the cysteine sulphydryl group with the carboxyl group-containing residue glutamate resulted in constitutive high levels of activity that may mimic the interaction with pyruvate (AFFOURTIT *et al.* 2002), while a change to the hydroxyl group-containing residue serine resulted in stimulation by succinate (BERTHOLD *et al.* 2000). Although some plants carry a naturally occurring allele with this cysteine to serine substitution (HOLTZAPFFEL *et al.* 2003), the mechanism by which this stimulation by succinate occurs is not known. The ability of the plant to alter enzymatic activity provides additional flexibility in rapidly responding to stress conditions (JUSZCZUK and RYCHTER 2003).

Plant alternative oxidase can be further stimulated by 5 mM glyoxylate, even after pyruvate stimulation (UMBACH *et al.* 2002). This stimulation requires $\mathrm{Cys_I}$ or a second conserved cysteine, $\mathrm{Cys_{II}}$ (UMBACH *et al.* 2006). This second conserved cysteine residue is found in one of the helices that form the diiron binding domain, but on the opposite side of the helix from the iron-coordinating motif. $\mathrm{Cys_{II}}$ is conserved in plant alternative oxidase, but not in the bacterial or fungal enzymes. Substitution of $\mathrm{Cys_{II}}$ with alanine

(Rhoads *et al.* 1998), glutamic acid or lysine increased basal enzyme activity in *A. thaliana* AtAOX1a expressed in heme-deficient *E. coli* (Umbach *et al.* 2006). The ability of Cys$_{II}$ to respond to glyoxylate stimulation was blocked in the presence of an oxidizing agent which created intersubunit disulphide bonds (Umbach *et al.* 2006). Both Cys$_{I}$ and Cys$_{II}$ play a role in modulation of plant alternative oxidase activity. It was suggested (Umbach *et al.* 2006) that because alternative oxidase produces less energy than the cytochrome-mediated ETC, and because replacing Cys$_{II}$ in plant enzymes increases basal activity in plants, conservation of this residue in plants may be important to ensure that the enzyme is not highly active except when specifically stimulated by molecules such as pyruvate and glyoxylate.

Plant alternative oxidases have been expressed in *Schizosaccharomyces pombe* where they confer cyanide insensitive respiration. When *Sauromatum guttatum* alternative oxidase was expressed in this yeast, its activity was constitutive and was not stimulated by the addition of pyruvate (Affourtit *et al.* 1999). Alternative oxidase from *A. thaliana* shares 54% identity with the *S. guttatum* enzyme (Joseph-Horne *et al.* 2001), but when expressed in *S. pombe* its activity was stimulated by pyruvate (Crichton *et al.* 2005). An alignment of plant alternative oxidase protein sequences was constructed using proteins known to be stimulated by pyruvate as well as proteins that are stimulated by succinate. Seven amino acid differences were seen between *S. guttatum* alternative oxidase and other plant alternative oxidases. Three of these substitutions are near Cys$_{I}$ and Cys$_{II}$ in the N-terminus, while one is in the C-terminus near the location of a site that when mutated reduces sensitivity to SHAM (Berthold 1998). The final three substitutions occur at consecutive residues in the N-terminus. Since the only difference between the *S. guttatum* and *A. thaliana* alternative oxidase proteins expressed in *S. pombe* is the amino acid sequence, differences in activity must be due to differences in the sequence. These data suggest that *S. gutattum* alternative oxidase is not stimulated by pyruvate, and that this is due to changes at specific sites in the protein. These changes may alter the conformation of the region around the conserved Cys_I with which pyruvate is thought to interact (CRICHTON *et al.* 2005).

1.13 Plant Alternative Oxidase Functions

Although the clearest physiological role of alternative oxidase is for generating heat necessary to volatilize aromatic compounds used to attract pollinators in the Voodoo Lily *S. gutattum* (VANLERBERGHE and MCINTOSH 1997), it appears to have other functions in plant growth and development. The two primary roles postulated for alternative oxidase are to balance metabolism during times of stress or active growth (BARTOLI *et al.* 2005; SIEGER *et al.* 2005), and to reduce production of oxygen free radicals (GRAY *et al.* 2004). It appears that there may even be separate signaling pathways to induce alternative oxidase in each of these conditions (GRAY *et al.* 2004).

1.13.1 Metabolism

The alternative pathway may be important for balancing the carbon cycle and electron transport (BARTOLI *et al.* 2005; SIEGER *et al.* 2005). At high cellular ATP concentrations electron flow through alternative oxidase will allow regeneration of oxidized electron carriers FAD and NAD, and thus continued production of carbon skeletons from the citric acid cycle (AFFOURTIT *et al.* 2001; VANLERBERGHE and

MCINTOSH 1997). The importance of alternative oxidase expression for normal growth was clearly demonstrated when antisense RNA of wild-type alternative oxidase was expressed in *A. thaliana*. After 21 days growth the antisense-expressing plants leaf areas were 27% smaller than wild-type plants, while plants over-expressing alternative oxidase had a leaf area 30% greater than wild-type (FIORANI *et al.* 2005).

The alternative pathway does not, however, act merely as an overflow for the cytochrome mediated ETC, since alternative oxidase can actively compete for electrons (AFFOURTIT *et al.* 2001). When alternative oxidase was expressed in *S. pombe,* which normally lacks this enzyme, growth rates were reduced (AFFOURTIT *et al.* 1999). This observation shows that less energy was available for growth, meaning that the alternative pathway, which produces less ATP than normal oxidative phosphorylation, was siphoning electrons away from the cytochrome-mediated ETC. Expression of alternative oxidase from another yeast, *H. anomala*, in *S. cerevisiae* also led to decreased growth rates (MATHY *et al.* 2006). The same study used 2-D gel analysis of mitochondrial proteins also to show an increase in Krebs cycle enzymes, indicating increased metabolic flux. Complex III was also upregulated, likely to better compete with alternative oxidase for electrons from the ubiquinone pool (MATHY *et al.* 2006). Since electron flow through alternative oxidase does not transport protons across the MIM, the proton gradient is decreased by its activity. To compensate, the ATP Synthase was downregulated, thus decreasing proton flow to the matrix and maintaining an elevated gradient (MATHY *et al.* 2006). These data confirm that alternative oxidase can actively compete for electrons in the ETC, and that its inappropriate activation can affect mitochondrial function and energy generation.

1.13.2 ROS

Reactive oxygen species such as H_2O_2, superoxide and hydroxyl radicals have been shown to be involved in alternative oxidase induction. These molecules can be produced if the ETC becomes saturated. For instance, ubiquinone can form a ubisemiquinone radical which produces superoxide (MAXWELL *et al.* 2002). By increasing the capacity of electron flow and preventing saturation of the ETC, alternative oxidase may reduce ROS formation (MAXWELL *et al.* 2002). ROS induction of alternative oxidase has been observed in a number of species such as *A. thaliana* (SWEETLOVE *et al.* 2002), *G. max* (DJAJANEGARA *et al.* 2002), *T. brucei* (FANG and BEATTIE 2003) and *H. capsulatum* (JOHNSON *et al.* 2003). In *N. tabacum* cells grown in the presence of antimycin A the ETC produces superoxide which is rapidly broken down into H_2O_2. Alternative oxidase expression is rapidly induced under these conditions (MAXWELL *et al.* 1999), although exogenous addition of the antioxidant flavone blocked induction after antimycin A treatment (VANLERBERGHE and MCINTOSH 1997). ROS may induce alternative oxidase indirectly by generally impairing mitochondrial function (MAXWELL *et al.* 2002), but some ROS such as H_2O_2 may also act directly as messengers. In *A. thaliana* H_2O_2 activates the MAP kinase ANP1 which initiates a phosphorylation cascade involved in stress signals (KOVTUN *et al.* 2000). While this particular system does not induce alternative oxidase it does show that ROS can be important in influencing nuclear gene expression.

Inhibition of alternative oxidase function in plants has been correlated with increased oxidative stress. *N. tabacum* cells expressing antisense alternative oxidase had increased mitochondrial ROS production (MAXWELL *et al.* 1999), as did *A. thaliana* plants expressing antisense alternative oxidase (UMBACH *et al.* 2005). When wheat leaves were incubated with SHAM in the dark, to prevent photosynthesis and ROS from chloroplasts, there was also an increase in ROS production (HAMMES *et al.* 2006). Part of the cellular response to ROS is increased production of antioxidant molecules such as ascorbate and glutathione. A change in the redox state of the pool of these molecules could also be a signal of ROS production (NOCTOR and FOYER 1998) and therefore induce alternative oxidase production.

1.14 Fungal Alternative Oxidase

Alternative oxidase is found in most fungi, *S. cerevisiae* and *S. pombe* being the two major exceptions (JOHNSON *et al.* 2003). In fungi there is generally a single structural gene, although a multigene family has been identified in *Candida albicans* where *aox1a* is expressed constitutively at low levels while *aox1b* is induced by cyanide and antimycin A (HUH and KANG 2001). Several important residues are conserved between the plant and fungal enzymes but there is fairly low overall identity between the plant and fungal proteins (24% between *S. gutattum* and *Magnaporthe griseae*) (JOSEPH-HORNE *et al.* 2001). The fungal enzyme functions as a monomer (UMBACH and SIEDOW 2000) and lacks the long N-terminal tail with its conserved cysteine $\mathrm{Cys_I}$ that is involved in regulation of the plant enzyme activity in some species. Alternative oxidase in fungi is therefore not inducible by α-keto acids (UMBACH and SIEDOW 2000), but is, however, stimulated by purine nucleotides (AFFOURTIT *et al.* 2002; HUH and KANG 2001; JOSEPH-HORNE *et al.* 2001; UMBACH and SIEDOW 2000).

1.14.1 Fungal Alternative Oxidase Role

The physiological role of alternative oxidase in fungi is not entirely clear. Although in *M. griseae* alternative oxidase transcription and enzymatic activity were induced by growth in the presence of H_2O_2 (YUKIOKA *et al.* 1998), *N. crassa* grown in the presence of H_2O_2 did not induce alternative oxidase activity (M. Chae, personal communication). In *Pichia membranifaciens* and *Debaryomyces hansenii* H_2O_2 induced alternative oxidase, but alternative oxidase activity did not change ROS levels (VEIGA *et al.* 2003b). The function of fungal alternative oxidase as a ROS protection system is therefore not clear.

Studies in *Podospora anserina* have implicated alternative oxidase in aging and senescence. Strains with nuclear gene mutations that impair or eliminate Complex IV function induced alternative oxidase, decreased ROS production and increased lifespan, with longer lifespan correlating with higher levels of alternative oxidase, and lower levels of cytochrome-mediated respiration (DUFOUR *et al.* 2000; OSIEWACZ and STUMPFERL 2001). In contrast, strains defective for Complex I and Complex IV of the ETC showed differences in lifespan, but these differences were not changed by the presence or absence of alternative oxidase, suggesting there is no causal link between alternative oxidase and lifespan (SELLEM *et al.* 2005). Over-expressing alternative oxidase in an otherwise wild-type strain had no impact on lifespan, while over-expressing alternative oxidase, and therefore increasing cellular energy generation, restored senescence and increased ROS production in a cytochrome respiration-deficient strain (LORIN *et al.* 2001). The function of alternative oxidase in senescence remains unclear.

A likely function of alternative oxidase in fungi is in pathogenesis. Over 800 plant species are known to produce cyanide compounds in response to wounding or pathogen attack (Veiga *et al.* 2003a) and alternative oxidase provides a means to overcome this defense mechanism.

1.15 *N. crassa* Alternative Oxidase

Alternative oxidase in *N. crassa* is a monomeric protein of approximately 35 kDa and is encoded by a single structural gene, *aod-1,* found on chromosome IV (Li *et al.* 1996). *aod-1* is not expressed under normal growth conditions but is induced when oxidative phosphorylation is disrupted by mutations in ETC components, inhibition of mitochondrial translation by chloramphenicol (CAP), inhibitors of the cytochrome-mediated ETC, inhibition of ATP synthase by oligomycin, and copper deprivation (Bertrand *et al.* 1983). Alternative oxidase activity can be detected within 30 minutes of addition of chloramphenicol to *N. crassa* liquid cultures (Edwards *et al.* 1974). AOD1 protein was first identified as alternative oxidase in *N. crassa* by observing a correlation between accumulation of a specific labeled protein and cyanide insensitive respiration when cells were grown in oligomycin in the presence of ^{35}S-sulphate (Bertrand *et al.* 1983). This was later confirmed immunologically using the *S. gutattum* alternative oxidase antibody (Lambowitz *et al.* 1989). The observation that mRNA is usually present only under inducing conditions (Lambowitz *et al.* 1989) suggests that the gene is controlled at the level of transcription. This was confirmed by nuclear run-on assays which showed that *aod-1* transcription is increased significantly after addition of an inducing agent (Tanton *et al.* 2003). However, uninduced wild-type cultures occasionally contain significant levels of *aod-1* mRNA but no AOD1 protein (DESCHENEAU *et al.* 2005; TANTON *et al.* 2003), suggesting that some post-transcriptional control mechanism does play a role in *aod-1* expression. There is evidence for post-transcriptional regulation of alternative oxidase in another filamentous fungus, *P. anserina*, where two strains accumulated equal amounts of alternative oxidase transcript, but very different amounts of alternative oxidase protein (BORGHOUTS *et al.* 2002). Although a second structural gene, *aod-3,* has recently been found in *N. crassa*, it does not appear to be expressed under conditions that induce *aod-1* (TANTON *et al.* 2003).

A gene that affects *aod-1* expression has also been identified (BERTRAND *et al.* 1983). This gene, called *aod-2,* appears to play a role in *aod-1* regulation since *aod-2* mutants fail to accumulate *aod-1* mRNA or protein (DESCHENEAU *et al.* 2005; LI *et al.* 1996). No similar locus has been identified in plants and *aod-2* has been the only additional gene required for alternative oxidase expression identified in any system.

1.16 Objectives of this Study

The purpose of this study was to identify components of the system responsible for inducing the nuclear gene *aod-1* in *N. crassa*. A reporter system was used in a mutagenesis screen to avoid isolating alleles of the structural gene *aod-1*. 15 putative regulatory mutants were identified by this screen. The mutants fell into 5 complementation groups affecting alternative oxidase and one chloramphenicol resistance gene. One mutant was cloned and identified as a Zn(II)2Cys6 transcription factor that may be involved in transcription of *aod-1*.

1.17 Zn(II)2Cys6 Binuclear Cluster Transcription Factors

Many fungal genes are regulated by a member of the large Zn(II)2Cys6 binuclear cluster transcription factor family. This class of transcription factor is specific to fungi (TODD and ANDRIANOPOULOS 1997). With one exception, all characterized zinc cluster proteins are transcriptional regulators (TODD and ANDRIANOPOULOS 1997); *CBF3* in *S. cerevisiae* encodes a protein that is part of the kinetochore (LECHNER 1994).

According to the *N. crassa* sequencing project, zinc cluster proteins are the most common transcription factor in *N. crassa* (BORKOVICH *et al.* 2004) where 77 open reading frames (ORFs) have been predicted to encode a member of this protein family. Of these ORFs, twenty showed no homology to yeast genes, nine had homologues in other filamentous fungi, and approximately half showed greatest similarity to another *N. crassa* zinc cluster protein (BORKOVICH *et al.* 2004).

1.17.1 Zinc Cluster Domain

This class of transcription factor binds DNA directly through the zinc cluster domain. This domain consists of six cysteine residues that coordinate two atoms of zinc. The consensus sequence for the zinc cluster domain is $CX_2CX_6CX_{5\text{-}16}CX_2CX_{6\text{-}8}C$ (LIU and MARZLUF 2004; TODD and ANDRIANOPOULOS 1997), and it is usually found near the N-terminus of the protein. One notable exception is Ume6p where the zinc cluster domain is found in the C-terminal portion of the protein (STRICH *et al.* 1994). Interestingly, this zinc cluster protein is a transcriptional repressor (STRICH *et al.* 1994), while nearly all other studied proteins are transcriptional activators or are bifunctional (SCHJERLING and HOLMBERG 1996; TODD and ANDRIANOPOULOS 1997). The zinc cluster recognizes a DNA sequence consisting of two CGG triplet repeats that are direct, inverted or everted, and separated by sequence of variable length and composition. Mutations in the cysteines of the zinc cluster have demonstrated that they are required for DNA binding and protein function (TODD and ANDRIANOPOULOS 1997).

DNA binding has been visualized in *GAL4* of *S. cerevisiae*, one of the best-studied zinc cluster proteins. X-ray crystallography identified the DNA recognition sequence of this transcription factor to be a 17-bp motif containing an inverted repeat CGG N_{11} CCG. The protein binds DNA as a dimer, with the zinc cluster of each member of the dimer binding to one repeat (MARMORSTEIN *et al.* 1992). Subsequent studies identified other similar motifs recognized by other members of this large fungal protein family. The Hap1p homodimer in yeast binds to a direct repeat CGG N_6 CGG (ZHANG and GUARENTE 1994), while Leu3p binds an everted repeat CCG N_{11} CGG (MAMANE *et al.* 1998).

The triplet repeats that comprise the DNA recognition sites are found in the promoters of the genes regulated by zinc cluster proteins. One study looked for targets of the *N. crassa* transcription factor *fluffy*. This gene encodes a protein that regulates the gene *easily wettable* (*eas*), a gene involved in conidiation. *fluffy* recognizes a CGG N_9 CCG motif and in *eas* this site is located in the promoter, 1498 bp upstream of the translational start site (RERNGSAMRAN *et al.* 2005). A search of the genome found 9000 of these motifs, of which 4600 were less that 2kbp upstream of the translational start site of an ORF (RERNGSAMRAN *et al.* 2005).

Interestingly, this same study showed that multiple potential binding sites might be present in a single promoter. Electrophoretic mobility shift assay (EMSA) experiments identified a major binding site at –1498 in the *eas* promoter, but also revealed two other weak binding sites (RERNGSAMRAN *et al.* 2005). The presence of multiple potential binding sites in the promoter has also been seen in the *Aspergillus nidulans* alcohol deydrogenase (*alcA*) promoter where binding at all sites appears to be necessary for full activation (PANOZZO *et al.* 1997).

1.17.2 Basic Region

Following the zinc cluster is often a short region enriched in basic residues (BAILEY and EBBOLE 1998; LIU and MARZLUF 2004). Although this motif has not been assigned a specific function, in at least one case it is known to enhance DNA binding by the zinc cluster domain (LIU and MARZLUF 2004).

1.17.3 Middle Homology Region

The so-called "middle homology region" (MHR) is found downstream of the zinc cluster domain, and is present in a number of zinc cluster proteins (SCHJERLING and HOLMBERG 1996). Its sequence is not highly conserved, and its precise function remains unknown. The MHR may play a role in regulation of transcriptional activation. Deletions in Leu3p (FRIDEN *et al.* 1989) and QA1F (GILES *et al.* 1991) that included the MHR resulted in constitutive activators. In contrast, deletion of the MHR in *fluffy* resulted in no transcriptional activation (RERNGSAMRAN *et al.* 2005). Point mutations in Cat8p revealed that the MHR is a site of phosphorylation by Snf1p, and that this phosphorylation regulates the protein's activity (CHARBON *et al.* 2004). The MHR may play a role in DNA binding site recognition and contribute to the sequence specificity of the transcription factor (SCHJERLING and HOLMBERG 1996).

1.17.4 Helical Domain

A common motif of zinc cluster proteins is a coiled coil or helical domain. In many proteins a coiled coil is formed by a leucine zipper motif not far downstream from the zinc cluster domain, while in other proteins a helical region is found near the C-terminus (SCHJERLING and HOLMBERG 1996; TODD and ANDRIANOPOULOS 1997). In most cases this helical domain is thought to play a role in dimerization. The *Aspergillus niger* protein XlnR has an unusual characteristic, in that the helical domain is necessary for nuclear localization. Although the protein has a predicted nuclear localization signal near the N-terminus, as is usual in zinc cluster proteins, deletions of the helical domain at the C-terminus resulted in a cytosolic localization (HASPER *et al.* 2004).

1.17.5 Activation Domain

The transcriptional activation domain of most zinc cluster proteins has not yet been defined (SCHJERLING and HOLMBERG 1996). A region in the C-terminus of Leu3p was found to be sufficient, in combination with a minimal DNA binding domain, for transcriptional activation, although regulation of that activation was lost (ZHOU *et al.* 1990). Deletions in the C-terminus of diverse zinc cluster proteins such as Gal4p (KEEGAN *et al.* 1986), XlnR (HASPER *et al.* 2004), and *fluffy* (RERNGSAMRAN *et al.* 2005), resulted in decreased activation. Many zinc cluster proteins contain a region of negative charge near the C-terminus, and activation domain locations often correlate with negatively charged regions (SCHJERLING and HOLMBERG 1996). Even when activation domains have been identified in zinc cluster proteins, their modes of action remain unknown.

1.17.6 Other Domains

In addition to the nearly universal motifs discussed above, there are many other domains seen in zinc cluster proteins. These motifs are often involved in the specific function of the protein and provide specific regulatory or activation functions necessary to the process in which the zinc cluster domain is involved (SCHJERLING and HOLMBERG 1996; TODD and ANDRIANOPOULOS 1997). An example is the PAS domain.

1.17.7 PAS Domains

A common protein motif found in some zinc cluster proteins is the PAS domain named for *Drosophila melanogaster* proteins period (PER), the vertebrate aryl hydrocarbon receptor nuclear translocator protein (ARNT) and the *D. melanogaster* protein single-minded (SIM) (PONTING and ARAVIND 1997; ZHULIN *et al.* 1997). Although these domains are conserved throughout evolution, with some highly conserved residues in the motif, it is the tertiary structure rather than the amino acid sequence that is most highly conserved. The existence of PAS domains is most often predicted by computer three-dimensional modeling, rather than sequence homology. The PAS domain folds into the PAS fold, which consists of 5 β sheets and an α helix.

The PAS fold has been annotated differently by different groups examining it. One group (ZHULIN *et al.* 1997) defined the PAS domain as consisting of two sensory box motifs S1 and S2 with a variable number of intervening residues. A second definition split the PAS fold into the PAS and PAC domains (PONTING and ARAVIND 1997), although these nearly correspond to the S1 and S2 boxes. Recent work has taken advantage of the large numbers of complete genome sequences now available. Predicted PAS domains were compared to a variety of solved 3D-structures of PAS fold-containing proteins. The results of this study confirmed that PAS and PAC domains are in fact two parts of a single protein domain which forms the PAS fold (HEFTI *et al.* 2004).

PAS domains are found in proteins from all kingdoms (ZHULIN *et al.* 1997) and often play a role in signal transduction. They commonly function as oxygen or redox sensors, both in bacterial two-component regulatory systems and in eukaryotic transcription factors (ZHULIN *et al.* 1997). PAS domains are also found in light-sensing proteins involved in circadian regulation.

One way in which the signal transduction function of PAS domains can be achieved is through ligand binding. A variety of ligands have been shown to bind to PAS domains including hemes and flavins (TAYLOR and ZHULIN 1999) to respond to redox signals. The signal transduction mode of action of most PAS domains is unknown, but a few examples have been examined in some detail. The bacterium *Sinorhizobium melilofi* FixL/FixJ two-component signaling system regulates nitrogen fixation genes in response to oxygen levels during symbiosis (TUCKERMAN *et al.* 2001). The FixL PAS domain interacts with a heme cofactor and binds oxygen. When oxygen dissociates from the PAS domain the conformation of that domain changes, leading to FixL autophosphorylation and activation of the protein. Phosphorylated FixL transfers the phosphate to FixJ, which then acts as a transcriptional activator (TUCKERMAN *et al.*

2001). The blue-light photoreceptor photoactive yellow protein has a chromophore cofactor (GENICK *et al.* 1997). When this chromophore is excited it alters its conformation, resulting in a slight alteration in the protein's conformation leading to changes in hydrogen bonding and protein shape (GENICK *et al.* 1997).

A well-characterized system in *N. crassa* that depends on PAS domain signal transduction is the regulation of circadian rhythm by the proteins *white collar 1* and *white collar 2* (WC1 and WC2). These proteins are both zinc finger transcription factors and each contains a PAS domain (BALLARIO *et al.* 1996; LINDEN and MACINO 1997). The PAS domain of WC1 binds a flavin ligand which mediates the blue-light response of the circadian rhythm (FROEHLICH *et al.* 2002; HE *et al.* 2002). Deletion of the PAS domain in WC1 abolishes the response to light exposure (HE *et al.* 2002). The PAS domains of WC1 and WC2 may also be important for the formation of the heterodimer white collar complex (WCC). WC1 and WC2 are capable of forming homo or heterodimers *in vitro* through PAS domain interactions (BALLARIO *et al.* 1998) and the WCC is present *in vivo* in light and dark conditions (TALORA *et al.* 1999). In response to blue light signals, the WCC binds the light response element (LRE) in the promoters of genes involved in the light response such as frequency (*frq-1*) and genes involved in carotenoid synthesis (HE *et al.* 2005).

The activity of the WCC is also regulated in two different ways by phosphorylation. WC1 is hyperphosphorylated after light exposure (SCHWERDTFEGER and LINDEN 2000) and in this state, loses its affinity for the *frq-1* promoter (SCHAFMEIER *et al.* 2005). In contrast, the hypophosphorylated WC1 strongly binds the *frq-1* promoter (SCHAFMEIER *et al.* 2005). A second phosphorylation occurs immediately downstream of the zinc finger domain of WC1 (HE *et al.* 2005). This phosphorylation is independent of light exposure, and appears to be constitutive. Mutations in these phosphorylation sites did not affect the ability of the WCC to respond to blue light, but they did affect transcription of *frq-1*.

Another example of PAS domain dimerization is in helix-loop-helix transcription factors. Recent examination of the interaction between ARNT and the dioxin receptor (DR) showed that the PAS domain was important for dimerization of these two proteins (CHAPMAN-SMITH *et al.* 2004). The role of the PAS domain in determining DNA binding was also assessed. When ARNT was expressed in *E. coli*, it was capable of forming homodimers that were able to bind target DNA sequences. When the PAS domain of ARNT was substituted for the PAS domain of DR in a DR chimera, binding affinity for the target sequence dropped (CHAPMAN-SMITH *et al.* 2004). This shows that PAS domains can also influence DNA binding, as well as dimerization and signal transduction.

1.17.8 Zinc Cluster Protein Transcriptional Regulation

There does not appear to be a common regulatory mechanism for zinc cluster proteins. Some, like *fluffy*, are expressed during specific times of development (BAILEY and EBBOLE 1998), while others, like *pco-1* which is involved in purine catabolism, are expressed constitutively (LIU and MARZLUF 2004).

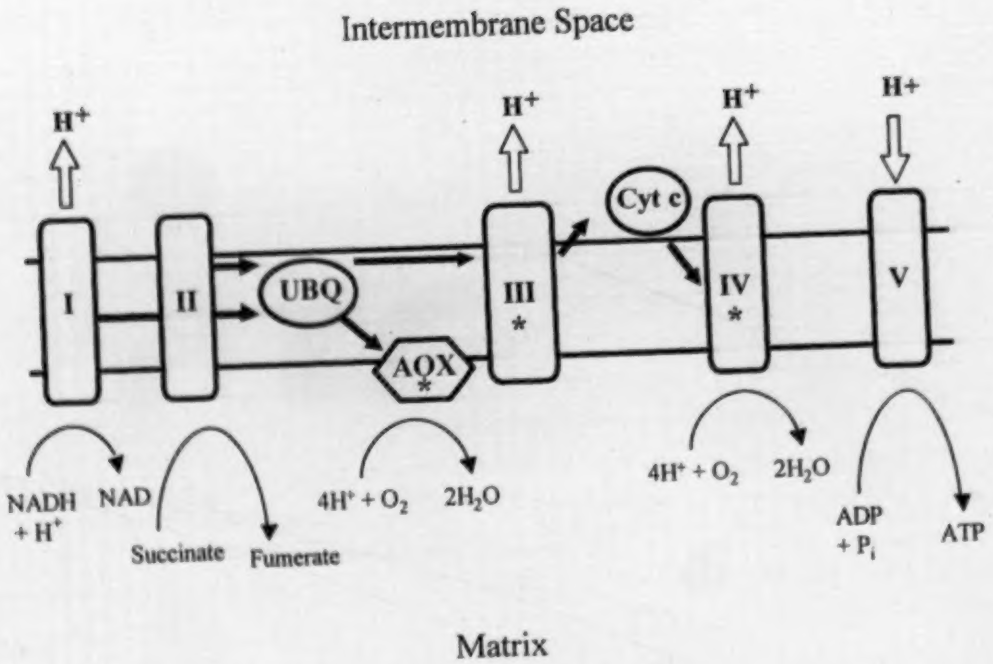
1.17.9 Zinc Cluster Protein Post Translational Regulation

There is evidence for specific post-translational modifications which regulate the activity of some zinc cluster proteins. Sip6p in *S. cerevisiae* is phosphorylated in starving cells (LESAGE *et al.* 1996). The kinase Snf1p is necessary for a response to glucose deprivation. *In vitro* experiments showed that Snf1p directly interacts with Sip6p and that phosphorylation is necessary *in vivo* for activation of the transcription factor (LESAGE *et al.* 1996). This experiment shows that Snf1p is necessary in the glucose deprivation response to activate a specific transcription factor.

1.18 Overview of the Project

The work in this thesis describes the isolation of five new loci necessary for induction of alternative oxidase in *N. crassa*. I characterized four of these mutants, and examined two of them more extensively. The *aod-5* mutation was found to affect a zinc cluster transcription factor. The *aod-6* mutant has a complicated phenotype. Trying to understand the nature of this mutant led to an examination of how AOD1 protein is assembled at the MIM.

Figure 1. The Electron Transport Chain. Electrons (black arrows) flow through the complexes of the electron transport chain. Electrons are donated to complexes I and II by the carriers NADH+$H^+$and succinate respectively. Electrons then pass to ubiquinone (UBQ) which can donate electrons to Complex III or to alternative oxidase (AOX). Alternative oxidase donates electrons to molecular oxygen to form water. Complex III donates electrons to cytochrome *c* (Cyt c), which then donates them to Complex IV. Complex IV is a terminal oxidase which donates electrons to molecular oxygen to form water. Complexes I, III and IV are sites of proton pumping (upward-pointing white arrows) from the matrix to the intermembrane space. Protons (downward-pointing white arrow) then flow back through complex V (ATPase) to convert ADP to ATP. Asterisks indicate potential sites of inhibition of electron flow. Alternative oxidase is inhibited by salicylhydroxamic acid, Complex III is inhibited by antimycin A and complex IV is inhibited by cyanide.


Intermembrane Space
H+
H+
H+
H+
Cyt c
I
II
UBQ
III
*
IV
*
V
AOX
*
NADH
+ H+
NAD
Succinate
Fumerate
4H+ + O2
2H2O
4H+ + O2
2H2O
ADP
+ Pi
ATP
Matrix

2. Materials and Methods

2.1 Growth of *N.crassa*

N.crassa was grown and handled as previously described (DAVIS and DE SERRES 1970). Alternative oxidase was induced by growth in the presence of chloramphenicol (2 mg/ml) or antimycin A (0.5 µg/ml). To measure growth rates, 10 µl of conidial suspensions containing 2 x 10^7, 2 x 10^6, 2 x 10^5, 2 x 10^4 conidia/ml were spotted onto plates containing standard sorbose media with appropriate supplements.

2.2 Strains

Strains used in this study are listed in Table 1.

2.3 Oligonucleotides

Oligonucleotides used in this study are listed in Table 2.

2.4 Plasmids

Plasmids used in this study are listed in Table 3.

2.5 Tyrosinase Reporter Assay

A volume of conidial suspension estimated to give 50-100 colonies was spread on each plate. Colonies grown under non-inducing conditions were allowed to grow for 2-3 days at 30°C. Colonies grown under inducing conditions, that is, in the presence of chloramphenicol, were allowed to grow for 3-4 days. After growth, colonies were overlayed with 10 ml freshly prepared solution of 10 mM tyrosine, 0.1% Triton-X-100. Plates were placed at 30°C and monitored regularly for colour change from white to brown colonies, indicating reporter activity. Conidia from the reporter strain began to change colour after 2 h. Colonies were scored after 5-7 h incubation with the tyrosine solution.

2.6 Ethyl methanesulfonate (EMS) mutagenesis

Conidia from 7-10 day old cultures were harvested through sterile cheesecloth, washed once in sterile dH_2O, and resuspended in 67 mM phosphate buffer pH 7.0 at a concentration of 2×10^7 conidia/ml. EMS was added to a final concentration of 0.15-0.3 M, and conidia were shaken at room temperature for five h. After EMS treatment conidia were washed twice with sterile 1X Vogel's solution. Mutants were subsequently selected by two methods. In the first method, called "Direct Selection," conidia were spread onto plates containing chloramphenicol at a dilution to obtain 50-100 viable colonies per plate. Colonies were formed by incubating the plates at 30°C for four days. The plates were then subjected to the tyrosinase assay. Colonies that remained white were picked for further analysis. The second method, called "Filtration Enrichment," used an enrichment mechanism to facilitate removal of undesirable strains prior to screening by the tyrosinase assay. 2×10^7 EMS treated conidia were grown with shaking in 500 ml liquid medium containing antimycin A (1.0 µg/ml) in a 2 L baffled flask for four days. The cultures were filtered every 24 h through sterile nylon stocking to remove any growing conidia, thereby enriching the culture for conidia sensitive to antimycin A. After four days the remaining conidia were plated on plates containing antimycin A. Colonies were formed by incubating the plates at 30°C for four days and the plates were then subjected to the tyrosinase assay. Colonies that remained white were picked for further analysis.

2.7 Conidial DNA Preparation for PCR

A small pea sized amount of conidia was mixed with 100 μL of cracking buffer (1 M sorbitol, 20 mM EDTA, 9 mg/ml β-D-glucanase enzyme, (Interspex Prodcuts Inc, San Mateo CA)). The mixture was incubated at 37°C for 10 min and then spun in a microcentrifuge for 10 min. The pellet was washed with 500 μl 1 M sorbitol, 20 mM EDTA and then resuspended in 100 μl sterile dH_2O. This mixture was then subjected to a standard glassmilk DNA isolation procedure (Geneclean II kit, Q Biogene, Carlsbad, CA) and eluted with 50 μl of sterile dH_2O.

2.8 Cytochrome Spectra

Spectra were obtained as described (BERTRAND and PITTENGER 1969). Mitochondrial preparations in 250 to 500 μl SEMP (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, 0.2 mM freshly prepared phenylmethylsulphonyl fluoride (PMSF)) containing 2 to 10 mg of protein were brought up to a volume of 2 ml with 2.5 % deoxycholate (in 10 mM Tris-HCl, 5 mM EDTA). The samples were mixed by inversion and then spun in a microcentrifuge at 13,000 rpm for 2 min. The supernatant was divided equally into two cuvettes. Spectra were obtained from 500 to 650 nm using a Shimadzu UV-Visible Recording Spectrophotometer, Model UV-265 (Shimadzu, Guelph, ON). First, a base line comparison between the two halves of the sample was taken. Following that, a few crystals of potassium ferricyanide were added to fully oxidize the reference, and a few crystals of sodium hydrosulphite were added to fully reduce the sample. Both samples were mixed gently by inversion until the crystals had dissolved. The spectrum was then taken comparing the different absorbances for the reduced and oxidized samples.

2.9 Antibody Production

Attempts were made to raise antisera against AOD5 fusion proteins purified by NiNTA chromatography. Fusion proteins were constructed which consisted of 114 amino acids of the C-terminus, and 96 amino acids near the middle of AOD5 fused to histidine tagged full length mouse dihydrofolate reductase (DHFR). PCR fragments encoding amino acids 304-400 or 617-730 of AOD5 were cloned into the *Bgl*II and *Xma*I sites of pQE40 (Qiagen, Mississauga, ON). These plasmids were transformed into *E. coli* strain BL21 for expression of the fusion proteins.

Following expression in *E. coli*, the fusion proteins were purified on a NiNTA column (Qiagen, Missisauga, ON) in 8 M urea according to the manufacturer's instructions, with the exception of the elution, which was performed with 0.1% SDS, 10mM Tris-HCl, pH 7.4. The eluate was injected into rabbits or guinea pigs. For the rabbits, the first injection was 1 mg of purified protein at a concentration of 1.33 mg/ml with an equal volume of Freund's Complete Adjuvant (Difco, Kansas, MO). Subsequent injections were 500 μg fusion protein at a concentration of 1mg/ml with an equal volume of Freund's Incomplete Adjuvant (Difco, Kansas MO). For guinea pigs, a similar procedure was followed, but the first injection was 0.2 mg purified protein and subsequent injections were 0.1 mg purified protein.

Rabbits were injected five times, with test bleeds taken after injections 3, 4 and 5. Guinea pigs were injected five times with test bleeds after injections 3 and 5. Blood was allowed to clot at room temperature for approximately two h, after which the sample was spun in a clinical centrifuge to pellet the clot. The serum supernatant was removed to a fresh tube and spun in a microcentrifuge to further clean the serum. The supernatant was once more removed to a fresh tube and heated at 50°C for 30 min. This potential antiserum was used in a milk buffer at a dilution of 1/500 for Western blot analysis.

2.10 Nuclei Isolation

Nuclei were isolated as described in (TANTON *et al.* 2003) with the following modifications. 10 to 15 g of harvested mycelium were ground to a powder under liquid nitrogen using a mortar and pestle. This powder was resuspended in nuclear isolation buffer (10 mM Tris-HCl pH 7.0, 10 mM EDTA, 4 mM spermidine, 1 mM spermine, 100 mM KCl, 0.1% β-Mercaptoethanol, 0.5% Triton X-100, 17% sucrose plus PMSF) to a volume of 45 ml. When thawed, the mixture was filtered through MiraCloth (Calbiochem, La Jolla CA). The filtrate was spun in a centrifuge at 6,500 rpm for 20 min. The supernatant (cytosolic fraction) was either discarded, or saved for further analysis and the remaining nuclear pellet was resuspended in 100-300 μl whole cell extract buffer (20 mM HEPES, 100 mM KCl, 5 mM DTT, 2 mM EDTA). This mixture was spun for 5 min in a microcentrifuge and the supernatant containing nuclear proteins retained. Fractions were assayed for the presence of histones by Western analysis using antiserum against *S. cerevisiae* bulk histone H3 (from M. Schultz).

2.11 RNA isolation

Mycelium was harvested by vacuum filtration and a portion of the pad was immediately wrapped in aluminum foil, frozen in liquid nitrogen, and stored at –80°C until needed. Pieces of approximately 100 mg were ground in liquid nitrogen using a mortar and pestle. RNA was isolated using either an RNeasy Plant Mini Kit (QIAGEN, Mississauga, ON) or hot phenol extraction as described previously (VERWOERD *et al.* 1989) with the following modifications for the latter procedure. After grinding in liquid nitrogen, the mycelial powder was mixed with 500 μl of fresh extraction mix (0.1 M LiCl, 100 mm Tris-HCl, pH 8.0, 10mM EDTA, 1% SDS, 50% phenol) at 65°C. This solution was mixed with 250 μl of 24:1 chloroform:isoamylalcohol and then centrifuged in a microcentrifuge at 13,000 rpm for 5 min at 4°C. The aqueous phase was taken to a clean tube and mixed with an equal volume of 4 M LiCl. This mixture was cooled at –20°C for 1 h and then centrifuged for 15 min at 4°C. The pellet was dissolved in 250 μl diethylpyrocarbonate (DEPC)-treated distilled water and mixed with 25 μl of 3 M sodium acetate, pH 5.2, and 550 μl 95% ethanol. RNA was allowed to precipitate at –20°C for 15 min whereupon the sample was spun at 13,000 rpm for 15 min at 4°C. The pellet was washed with 50 μl 70% ethanol and then dissolved in 50 μl DEPC-treated water.

2.12 RT-PCR

First strand synthesis of cDNA was performed with Superscript II reverse transcriptase (Invitrogen, Burlington, ON) following manufacturer's directions. 3 µg total RNA was used as template and an oligo-dT (dT20) oligonucleotide was used to prime the reaction. Following first strand synthesis 2 µl (1/10 of the reaction volume) was used for PCR. This reaction was carried out in the presence of 5% dimethyl sulphoxide (DMSO).

2.13 PCR Fragment Purification

The desired DNA band was cut from an agarose gel and transferred to a 1.5 ml eppendorf tube where it was dissolved in three volumes 6 M sodium iodide at 55°C. This solution was spun through a mini-prep column (Qiagen, Mississauga, ON) for 1 min at 5,000 rpm in a microcentrifuge to allow DNA to bind the matrix in the column. Aliquots (up to a maximum of 750 µl) were applied to the column before spinning. The column was washed with 500 µl 6 M sodium iodide by spinning for 1 min at 5,000 rpm in a microcentrifuge. A second wash was performed with 750 µl buffer PE (10mM Tris-HCl pH 8 in 50% ethanol) spun through the column for 1 min at 5,000 rpm in a microcentrifuge. The column was then spun for 1 min at 10,000 rpm in a microcentrifuge to remove any residual ethanol. DNA was eluted from the column into a fresh 1.5 mL eppendorf tube by incubating the column with 30 to 50 µL of water, followed by a 1 min spin at 13,000 rpm in a microcentrifuge.

2.14 PCR Mutagenesis

Changes to the *aod-5* coding sequence were introduced using a PCR mutagenesis system based on the QuikChange Multi site-directed mutagenesis method (Stratagene, La Jolla, CA) according to the instructions of the manufacturer with some modifications. For deletions and amino acid substitutions pBNA5-2 was used as a template and Pfu polymerase was used for elongation. For insertion of a his-tag at the N-terminus of *aod-5* pBaod5 was used as a template and a 100:1 Taq:pfu mix was used for elongation.

2.15 *N. crassa* transformation

DNA was transformed into *N. crassa* by electroporation of conidia as previously described (MARGOLIN *et al.* 1997; MARGOLIN *et al.* 2000) with some modifications. Conidia between three and ten days old were harvested in cold, sterile water and filtered through sterile cheesecloth. The conidia were washed three times with 50 ml cold, sterile 1 M sorbitol and resuspended to a final concentration of 2.0 to 2.5 x10^9 conidia/ml. 2 µg of linearized plasmid DNA at a concentration of 0.4 µg/µL was mixed with 40µL of conidia and placed on ice in electroporation cuvettes (2 mm gap) for 5 min. Electroporation was performed at 1.5 kV, 480 Ω on an Electro Cell Manipulator 600 (Harvard Apparatus, Holliston, MA) with time constants between 20 and 21 ms. Immediately following the pulse, 1 ml of cold, sterile 1M sorbitol was added and the conidia were incubated at 30°C for 60 min to recover. Aliquots of between 5% and 50% of the total volume of the electroporated conidia were added to 50 ml top agar at 46-48°C (standard sorbose solid medium containing 1 M sorbitol (DAVIS and DE SERRES 1970)) plus the appropriate antibiotics and nutritional requirements for selection of the transformants. After gentle but thorough mixing, the mixture of top agar and conidia was spread onto 5 plates of the same medium, but without 1 M sorbitol, and incubated at 30°C until colonies formed.

2.16 Purification of transformants

Pure homokaryotic transformed strains are desired after electroporation, and to this end colonies were subjected to a purification procedure. Single transformant colonies were picked using sterile glass Pasteur pipettes and transferred to slants containing Vogel's medium with appropriate nutritional supplements and the selective antibiotic at half the normal concentration. Use of full strength antibiotics has been observed to inhibit conidiation. Slants were incubated at 30°C until mycelium had covered the medium, at which point they were placed at room temperature to conidiate. Conidia were streaked for single colonies onto plates identical to those used for plating the electroporation, and incubated at 30°C until colonies formed. Single colonies were picked to slants without antibiotics for growth and conidiation.

2.17 *In vitro* import

Mitochondria for *in vitro* import studies were isolated as described (MAYER *et al.* 1995) and import of mitochondrial preproteins was performed as described (HARKNESS *et al.* 1994). Preproteins were produced by coupled transcription and translation in rabbit reticulocyte lysate (Promega TnT reticulocyte lysate system, Madison WI) in the presence of [^{35}S]-methionine (ICN Biochemicals, Costa Mesa, CA) for the indicated time points at the indicated temperatures. Some import reactions were followed by a carbonate extraction (see below). Import reactions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and viewed by autoradiography or a phosphorimager system using the analysis program Imagequant (version 5.2 Molecular Dynamics).

2.18 Carbonate Extraction of Proteins

20-50 µg of mitochondria were suspended in 1 ml cold 0.1 M sodium carbonate (Na_2CO_3), pH 11.5 and incubated on ice for one h. The mixture was then spun at 45,000 rpm for one h in an ultracentrifuge to pellet the membranes. Proteins from the supernatant were precipitated with a trichloroacetic acid (TCA) precipitation (see below). Both pellets were solubilized in cracking buffer (0.06 M Tris-HCl pH 6.7, 2.5% SDS, 0.01% β-mercaptoethanol, 5% sucrose). The mixture was shaken for 5 min, boiled for 5 min, and then subjected to SDS-PAGE.

2.19 Sonication of Mitochondria

50 µg of mitochondria were suspended in 1 ml cold SEM and subjected to a sonication regimen. Samples were sonicated on ice with a Sonic Dismembrator (Fisher, Nepean, ON) using the micro-tip set at 35% for 1, 2, 4, or 6 ten-second bursts. Samples sonicated for more than one burst sat on ice for 10s between bursts. One sample was not sonicated, but was subjected to subsequent treatments as a control. After sonication the mixture was spun at 45,000 rpm for one h in an ultracentrifuge to pellet the mitochondria and membranes. Proteins from the supernatant were precipitated with trichloroacetic acid (TCA) precipitation (see below). Both pellets were solubilized in cracking buffer (0.06

M Tris-HCl pH 6.7, 2.5% SDS, 0.01% β-mercaptoethanol, 5% sucrose. The mixture was shaken for 5 min, boiled for 5 min, and then subjected to SDS-PAGE.

2.20 TCA Precipitation

TCA (72% w/v) was added to the protein solution to a final concentration of 15%. Samples were incubated overnight at 4°C or on ice for 30 min, following which proteins were pelleted by a 15 min spin at13,000 rpm in a microcentrifuge. The supernatant was discarded and 1 ml acetone was added. The tube was then shaken for 5 min and spun for five min at13,000 rpm in a microcentrifuge. The supernatant was removed and the pellet was dried for 30 min at 37°C. Proteins were solubilized in cracking buffer, shaken for 5 min, boiled for 5 min, and then subjected to SDS-PAGE.

2.21 Creation of plasmid pBNA5

Cheryl Nargang subcloned a 3.5 kbp *XbaI* fragment of cosmid pMOcosX14F7 into another *Xba*I fragment from the same digest that contained the original pMOcosX vector to create a plasmid called ba#11. I excised and isolated the 3.5 kbp *Xba*I fragment and cloned it into the *Xba*I site of the pBSKII multiple cloning site (MCS) to form pBaod-5. Plasmid pTom22His was digested with *Not*I to generate a fragment of approximately 2.2 kbp that contained the gene which confers benomyl resistance. This fragment was cloned into the *Not*I site in the MCS of pBaod-5 to create plasmid pBNA5 containing benomyl resistance and the *N. crassa* genomic *aod*-5 cloned into pBSKII.

2.22 Creation of plasmid pBNAsp5

Using primers ICL34 and ICL35 I amplified a fragment of approximately 3 kbp from *A. nidulans* gDNA (from M. Chae). This band was purified, digested with *XbaI* inserted into the *Xba*I site of the pBSKII multiple cloning site (MCS) to form pBAsp5. Plasmid pBNA5-2 was digested with *Not*I to generate a fragment of approximately 2.2 kbp that contained the gene which confers benomyl resistance. This fragment was cloned into the *Not*I site in the MCS of pBAsp5 to create plasmid pBNAsp5 containing benomyl resistance and the *A. nidulans* genomic *aod*-5 cloned into pBSKII.

2.23 Other Techniques

The following procedures were employed according to the manufacturer's instructions or previously described procedures: isolation of plasmid or cosmid DNA (Qiagen, Missassauga, ON), genomic DNA extraction (WENDLAND *et al.* 1996), automated sequencing using a DyeNamic sequencing kit (Amersham Biosciences) with a model 373 stretch sequencer separation system (Applied Biosystems, Foster City, CA), protein determination with the Coomassie dye binding assay (Bio-Rad Hercules, CA), separation of proteins by SDS-PAGE (LAEMMLI 1970), Western blotting (GOOD, 1989), Western blot ECL detection using LumiGLO chemiluminescent substrate (Kirkegaard and Perry Laboratories, Gaithersburg MD). Radioactive precursor proteins for import were generated by coupled *in vitro* transcription and translation with the Promega (Madison, WI) TNT reticulocyte lysate system in the presence of [^{35}S]-methionine (ICN Biochemicals, Costa Mesa, CA).

Table 1.

Strains used during this study.

Strain	Source	Genotype	Mating type	Notes
		Control Strains		
NCN233		*pan-2, A*	A	pan-2
NCN235		*pan-2, a*	a	pan-2
NCN251-5	FGSC #2849	*A*	A	
763	FGSC	*nic-1, A*	A	nic-1
764	FGSC	*nic-1, a*	a	nic-1
7262 (MII)	FGSC	*pyr-3, trp-3, am*$_{112}$ *inl, inv, mei-2*		trp-3
		Mapping Strains		
997	FGSC	*alcoy, A*	A	*alcoy*, for mapping
998	FGSC	*alcoy, a*	a	*alcoy*, for mapping
1205	FGSC	*al-1, arg-5, A*	A	for testing linkage between LG I and LG II
1206	FGSC	*al-1, arg-5, a*	a	for testing linkage between LG I and LG II
1208	FGSC	*trp-1, ylo, a*	a	for testing linkage between LG III and LG VI
1243	FGSC	*inl, cot-1, A*	A	for testing linkage between LG IV and LG V
1244	FGSC	*inl, cot-1, a*	a	for testing linkage between LG IV and LG V
2283	FGSC	*un-5, al-2, arg-13, a*	a	for testing linkage to LG I
2997	FGSC	*pyr-4, arg-12, A*	A	for testing linkage to LG II
2998	FGSC	*pyr-4, arg-12, a*	a	for testing linkage to LG II
3753	FGSC	*caf-1, al-3, his-6, a*	a	for testing linkage to LG V
3789	FGSC	*ro-10, al-2, un-18, A*	A	for testing linkage to LG I
4120	FGSC	*acr-2, trp-1, dow, a*	a	for testing linkage to LG III
4121	FGSC	*cys-10, pdx-1, pan-1, A*	A	for testing linkage to LG IV
4122	FGSC	*cys-10, pdx-1, pan-1, a*	a	for testing linkage to LG IV
4141	FGSC	*nic-3, met-7, arg-10, A*	A	for testing linkage to LG VII
4142	FGSC	*nic-3, met-7, arg-10, a*	a	for testing linkage to LG VII
6563	FGSC	*ro-7, arg-5, rip-1, A*	A	for testing linkage to LG II

6564	FGSC	*ro-7, arg-5, rip-1, a*	a	for testing linkage to LG II
6808	FGSC	*trp-1, ylo-1, A*	A	for testing linkage between LG III and LG VI
7224	FGSC	*ro-10, nit-2, leu-3, A*	A	for testing linkage to LG I
7563	FGSC	*nit-6, ylo-1, un-23*	A	for testing linkage to LG VI
7564	FGSC	*nit-6, ylo-1, un-23*	a	for testing linkage to LG VI
		Strains Derived from Mutagenesis or Crosses		
7064 (NSBAN-4*a*)	H. Bertrand	*aod-2-4, nic-1, al-2, a*	a	aod-2 mutant
7207	H. Bertrand	*aod-1, pan-2, A*	A	aod-1 mutant
T11-76	A. Todd	*T, al-1, a + pBAT*	a	T1P11 with pBAT
AA1-15,	This Study	*aod-1, arg-5, A*	A	7207 X 1206
AA1-27, -37, -42	This Study	*aod-1, arg-5, al-1*	27, 37=A, 42=a	7207 X 1206
2-195	This Study	*E1, T, al-1, a + pBAT*	a	first EMS mutant
EN195-26, 44	A. Todd	*E1, pan-2, al-1, a, + pBAT*	a	2-195 X NCN233
EN195-34	A. Todd	*E1, pan-2, al-1, A, + pBAT*	A	2-195 X NCN233
EN195-109	A. Todd	*E1, pan-2, a*	a	2-195 X NCN233
4-294		*E2, T, al-1, a + pBAT*	a	second EMS mutant
EN294-46	A. Todd	*E2, pyr-6, A*	A	4-294 X NCN246
5-14	This Study	*E3, T, al-1, a + pBAT*	a	third EMS mutant
EN14-18	A. Todd	*E3, ad-2, al-1, a + pBAT*	a	5-14 X 7263
EN14-34	A. Todd	*E3, ad-2, al-1, A + pBAT*	A	5-14 X 7263
L1-6	This Study	*E5, T, al-1, a + pBAT*	a	fifth EMS mutant
PL16-56	This Study	*E5, pan-2 + pBAT, A*	A	L1-6 X NCN233
PL16-63	This Study	*E5, pan-2 + pBAT, a*	a	L1-6 X NCN233

				sixth EMS mutant
L1-13	This Study	*E6, T, al-1, a + pBAT*	a	L1-13 X NCN233
PL13-35, -77, -79	This Study	*E6, pan-2, a*	a	PL13-77 X NCN233
PL1377-24,	This Study	*E6, pan-2, a*	a	PL13-77 X NCN233
PL1377-51, -66	This Study	*E6, pan-2, A*	A	
				eighth EMS mutant
L2-25	This Study	*E8, T, al-1, a + pBAT*	a	L2-25 X 7267
EL25-6	A. Todd	*E8, met-7, al-1, A + pBAT*		L2-25 X 7267
EL25-27	A. Todd	*E8, met-7, a + pBAT*	a	L2-25 X 7267
EL25-37	A. Todd	*E8, met-7, A + pBAT*		
				nineth EMS mutant
L2-37	This Study	*E9, T, al-1, a + pBAT*	a	L2-37 X NCN233
PL37-20, -38	This Study	*E9, pan-2 + pBAT, a*	a	L2-37 X NCN233
PL37-1, -28	This Study	*E9, pan-2 + pBAT, A*	A	L2-37 X NCN233
PL37-117	This Study	*E9, pan-2*		L2-37 X 763
NL37-19, -31, -98	This Study	*E9, nic-1 + pBAT, A*	A	
				tenth EMS mutant
L2-40	This Study	*E10, T, al-1, a + pBAT*	a	L2-40 X NCN233
PL40-12, -35	This Study	*E10, pan-2 + pBAT, A*	A	L2-40 X NCN233
PL40-10, 23, 33	This Study	*E10, pan-2*	23=A, 33=a	L2-40 X 763
NL40-14, -30, -32	This Study	*E10, nic-1, a*	a	L2-40 X 763
NL40-5, -20,-33,-46	This Study	*E10, nic-1, A*	A	
				eleventh EMS mutant
L2-61	This Study	*E11, T, al-1, a + pBAT*	a	L2-61 X NCN233
PL61-5, -54	This Study	*E11, pan-2 + pBAT*	5=a, 54=A	L2-61 X NCN233
PL61-63	This Study	*E11, pan-2, a*	a	L2-61 X 763
NL61-130	This Study	*E11, nic-1, A*	A	L2-61 X 763
NL61-19, -119, -127 -128, -129, -130	This Study	*E11, nic-1, + pBAT*	A except 129	
				twelfth EMS mutant
L2-62	This Study	*E12, T, al-1, a + pBAT*	a	L2-62 X 7262
EL62-2, -25	A. Todd	*E12, trp-3, A*	A	

EL62-22	A. Todd	*E12, trp-3, a*	a	L2-62 X 7262
L2-64	This Study	*E13, T, al-1, a + pBAT*	a	thirteenth EMS mutant
EL64-46	A. Todd	*E13, ad-1, al-1, a + pBAT*	a	L2-64 X 7266
EL64-47	A. Todd	*E13, ad-1, A + pBAT*	A	L2-64 X 7266
L2-67	This Study	*E14, T, al-1, a + pBAT*	a	fourteenth EMS mutant
EL67-2	A. Todd	*E14, ad-3A, al-1, A + pBAT*	A	L2-67 X 7261
EL67-6, -33, -46	A. Todd	*E14, ad-3A, A + pBAT*	A	L2-67 X 7261
7-64	This Study	*E15, T, al-1, a + pBAT*	a	fifteenth EMS mutant
PN64-2, -9, -87	This Study	*E15, pan-2 + pBAT*	2, 87=A	7-64 X NCN233
PN64-69, 79, 91	This Study	*E15, pan-2*	69=a, 79, 91=A	7-64 X NCN233
NN64-29, 32	This Study	*E15, nic-1 + pBAT, A*	A	7-64 X 763
NN64-1, -17, -33	This Study	*E15, nic-1, A*	A	7-64 X 763
NN64-35	This Study	*E15, nic-1, a*	a	7-64 X 763
		aod-5 **Transformant Strains**		
A5R-1	This Study	E10, *pan-2 + pBNA5-2*		PL40-23 transformed with pBNA5
HisA5N	This Study	E10, *pan-2 +pHisaod-5N*		PL40-23 transformed with pHisaod5C
HisA5C	This Study	E10, *pan-2+ pHisaod-5C*		PL40-23 transformed with pHisaod5N
A5RAsp -1 2, 3, 5, 6	This Study	E10, *pan-2+ pBNAsp5*		PL40-23 transformed with pBNAsp5
		Import Strains		
HV (7255)	FGSC	*cyh-2 lys-2 leu-5 mei-2, a*	a	parent of oxa-1^{RIP} heterokaryon
ox80-15-1		*cyh-2 lys-2 leu-5 mei-2* $oxa1^{RIP}$ $oxa1^{RIP}$ *(EC) + am132 inl inv mei-2*		oxa-1^{RIP} sheltered heterokaryon

Table 2.

Primers used during this study.

Primer	Sequence (5' to 3')	Purpose
ICL1 [1]	ACGATGCCGGACGACGTTGG	sequence *aod-5*
ICL2	ACGACCATGCCAACGGTGC	sequence *aod-5*
ICL3	CTCCAGCGCTATTTGACCCG	sequence *aod-5*
ICL4	CGCCACCAACTACACGGGGG	sequence *aod-5*
ICL5	GAACCGGCGAAATAGCCGCC	sequence *aod-5*
ICL6	CTCCCACAGCTTCAGGGGG	sequence *aod-5*
ICL9	GCAGCAGGATTGTAGTTGGG	sequence *aod-5*
ICL11	TAGCGTCCCGGGACCCAGAACTGACTGTGGCCCC	cloning middle region for antibody production
ICL12	CGCACCCCCGGGCCGTCAGACACCACTTACG	cloning C-term region for antibody production
ICL14	TAGATGTGATCCTCTGGGCC	amplify reporter tyrosinase
ICL16	TCTGTATATATAGAGCTAAGGCACGATGCACCATCACCATCACCATCCGGACGACGTTGGACCCGCCG	insert N-terminal 6 His-tag
ICL17	TCTTAGAGATCTAGCGACTTCCTGACTCTGGATGCC	cloning middle region for antibody production
ICL18	CGATCGAGATCTCGACTTCTCAAGTACCGCACC	cloning C-term region for antibody production
ICL20	CTTTTTCCTCCTGGGCCGC	sequence *aod-5*
ICL22	GGAGAAAAGCTTCCAGGCCCTTTTCGAGCATG	delete Arg 474
ICL24	CGCTTGCCGTCGAACCGAAATAGCCGCCG	delete G 497
ICL26	CGCTTGCCGTCGAACGCCCGAAATAGCCGCCG	change G 497 to Ala
ICL27	AGGTGACGAACGACCATCCCAACGGTGCATCAAAC	change Cys 91 to Ser
ICL28	TATCATGATCAACGTAAGTGGTGTCCACCATCACCATCACCATTGACGGGCCTTTTCTCTTTTCGCCC	insert C-terminal 6 His tag
ICL29	CATGCACCAATCGCCACCGGCCATCGCTTGCC	delete Ser 488

ICL30	AGCCGCCGTTAACAAGTTCACGGCGCTAACAG	delete E 505
ICL34	TTGTCCTCTAGACCAAACGGTCTCTGTCTCCC	amplify *A nidulans aod-5*
ICL35	TAGCATTCTAGACACCTCTTGGCCTCGGTTGG	amplify *A nidulans aod-5*
ao2	AACGCCATGTTTCTCAGCTACC	sequencing *aod-1*
ao4	GATCCGAATCCGTTTGTGAGC	sequencing *aod-1*
ao5	TTAGTTGGGCCGCTTGTCC	*aod-1* PCR, sequencing
ao6	CCGGCCAAAGAGACCGCC	sequencing *aod-1*
ao7	ATAGCGGGCGCTAGCGAAAGG	sequencing *aod-1*
ao8	ACCAGCGCGGACAAACCT	sequencing *aod-1*
ao10	CATGAAGCCATTCGCAACTTATG	sequencing *aod-1*
ao12	TCACAAACCTCAAGCGAGTTCCAA	sequencing *aod-1*
ao21	AGTGATGTGAAGTTGTAATGGA	sequencing *aod-1*
ao23	TTGCCATTCCGTCCTCGTA	sequencing *aod-1*
ao25	ACACTAGACGCGTGTAA	sequencing *aod-1*
ADE19	GGAGGTAGAGATTGAACTGCTCCGG	amplify tyrosinase
ADE58	CTCGAGCCCAACACCAAGAACGTC	sequencing tyrosinase
ADE59	CGTACTTTGATTGGGCTAGTCAGCC	sequencing tyrosinase
ADE60	CCATGTGTATGTGGTCACTCCCC	sequencing tyrosinase
ADE61	TCTCGGCTGCTGCTGCTCATGCAG	sequencing tyrosinase
ADE62	CCTCGCTTTGCTGCAGGATATTGTG	sequencing tyrosinase
FNA88	CCTTCCCTCCAGAAGGCTTTATGCG	amplify *aod-1*
FNA 272	CAAGGAAGCTTTCTCCCAAGAAGCCCACCAAGCAGGACC	amplify Tim8 for RT-PCR
FNA 273	GGAACAAGCTTCAATCGCAACACATAACCCTGCCCAATAC	amplify Tim8 for RT-PCR

FNA 341	CAACGTCTACTAGTGGACGATATAAACGTCCCGTGTCTAGTGCT	amplify *aod-1* for probe
FNA 342	NNNNNNNNGAATTCCAGAGAGAAGAACCCAGTATCTTTTTACC	amplify *aod-1* for probe
FNA 412	GCAACGTCGCGGCCGCGCCCAACGCAAACATTCCGCATAACGAACG	amplify *aod-5* with 500 bp flanking sequence
FNA 413	CAACGTCTGCGGCCGCCCATATCTGGTCTCGCACGCATTGTGTTCC	amplify *aod-5* with 500 bp flanking sequence

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[1] Bold print indicates primers created in this study.

Table 3.
Plasmids used during this study.

Vector (alternate name)	**Source**	**Description**
pBSK(II)	Stratagene	
pAOcB	C. Nargang	*aod-1* cDNA+ bleomycin resistance, template for probe PCR
pBAT	A. Todd	3.3 kbp *aod-1* upstream sequence placed upstream of tyrosinase gene with bleomycin resistance cloned with *Xba I*
pMOcosX14F7		component of *N. crassa* genomic library
ba#11	C. Nargang	3.5 kbp *Xba* I digest fragment of pMOcosX14F7 cloned into pMOcosX vector
pBaod5	This Study	3.5 kbp *Xba* I digest fragment of ba#11 cloned into pBSKII
pTom7LR-benomyl	N. Go	HisTag version of Tom7, with benomyl resistance
pBNA5	This Study	pBaod5 with Benomyl resistance from pHISTom22 cloned into the *NotI* site
p16.1	This Study	pBaod5 with a partial his tag at the N terminus
pHisaod-5N(p16.11)	This Study	pBaod5 with a 6 His tag at the N terminus
pHisaod-5C		pBaod5 with a 6 His tag at the C terminus
pC91S (p27.1)	This Study	pBNA5 with mutant *aod-5* with a change of C91S
pR474 (p22.1)	This Study	pBNA5 with mutant *aod-5* with a deletion of R474
pS488 (p29.2)	This Study	pBNA5 with mutant *aod-5* with a deletion of S488
pG497 (p24.2)	This Study	pBNA5 with mutant *aod-5* with a deletion of G497
pG497A (p26.3)	This Study	pBNA5 with mutant *aod-5* with a change of G497A
pE505 (p30.9)	This Study	pBNA5 with mutant *aod-5* with a deletion of E505
pBAsp5	This Study	*A. nidulans aod-5* cloned in *Xba* I site of pBSKII
pBNAsp5	This Study	pBNAsp5 with Benomyl resistance from pBNA5 cloned into the *NotI* site
pQE40	Qiagen	expression vector
pM6-2	This Study	"middle region" of AOD5 in pQE40
pC2-1	This Study	C-terminal region of AOD5 in pQE40

3. Results

3.1 Isolating Alternative Oxidase Regulatory Mutants

Previous attempts have been made in other labs to isolate alternative oxidase regulatory mutants by conducting mutagenesis screens based on selection of mutants sensitive to chloramphenicol or antimycin A (BERTRAND *et al.* 1983; EDWARDS *et al.* 1976). These screens resulted in the isolation of many alleles of the structural gene *aod-1* and three alleles of the known regulatory locus *aod-2* (BERTRAND *et al.* 1983; EDWARDS *et al.* 1976). To reduce the likelihood of generating further alleles of the structural gene, our lab decided that use of a reporter system would be more likely to isolate regulatory mutants.

3.1.1 The Reporter System

Andrea Todd (née Descheneau), formerly a graduate student in our lab, developed a reporter system consisting of the *N. crassa* tyrosinase structural gene fused to 3 kbp of sequence upstream of the *aod-1* structural gene. This reporter gene was cloned into a plasmid containing the gene conferring resistance to bleomycin, for selection of transformants, to form the final plasmid called pBAT for bleomycin resistance *aod-1* and tyrosinase. Andrea created an *N. crassa* reporter strain by transforming a tyrosinase mutant strain (T1P11) with this reporter construct. This transformation resulted in integration of the reporter at an ectopic chromosomal location leaving the endogenous alternative oxidase gene intact. Because the tyrosinase enzyme is involved in melanogenesis a visual colour assay could be performed with this reporter as tyrosinase converts colourless, exogenous tyrosine into a dark precipitate (KOTHE *et al.* 1993).

3.1.2 Mutagenesis of the Reporter Strain

In *N. crassa* alternative oxidase can be induced by treatment with various inhibitors that reduce the effectiveness of oxidative phosphorylation. For example, chloramphenicol inhibits mitochondrial translation, which in turn prevents efficient formation of oxidative phosphorylation complexes containing mitochondrially encoded subunits. Antimycin A and cyanide specifically inhibit complexes III and IV of the ETC, respectively (BERTRAND *et al.* 1983; LI *et al.* 1996). Andrea and I performed EMS mutagenesis (DAVIS and DE SERRES 1970) of conidia of the reporter strain in the hope of generating mutants that lacked tyrosinase activity under conditions that induced alternative oxidase. EMS is a chemical mutagen that produces primarily point mutations.

Two methods were used to mutagenize conidia from the reporter strain. Method 1 was called "Direct Selection". After EMS mutagenesis, conidia were plated directly on plates containing chloramphenicol. Method 2 was called "Filtration Enrichment". In this method mutagenized conidia were incubated in liquid medium containing antimycin A for several days. Each day the culture was filtered to remove germinating conidia. Since the culture contained antimycin A, only conidia with alternative oxidase activity would be able to grow, since the flow of electrons could bypass the inhibited Complex III. Filtering out the conidia that were able to grow and produce hyphal extensions reduced the number of conidia to be screened, and enriched for alternative oxidase deficient conidia. After filtration, conidia were spread on plates containing chloramphenicol. For both methods, plates were incubated until colonies formed. Next, a tyrosine solution was added to the plates, and after several hours colonies were screened on the basis of colour. A change from white to brown indicated tyrosinase activity and a functional system for alternative oxidase induction. Colonies that remained white were picked for further study as potential alternative oxidase regulatory mutants that were unable to induce the reporter.

To confirm that these strains were defective for alternative oxidase induction they were individually re-tested using the tyrosinase plate assay (Figure 2). Those strains that were confirmed to be non-inducing using this test were further examined using an oxygen respirometer for *bona fide* alternative oxidase activity under inducing conditions. When grown under inducing conditions wild-type strains express the *aod-1* gene and therefore continue to respire when cyanide is added to a sample of mycelium (they are insensitive to cyanide) (Figure 3). Mutants that cannot induce *aod-1* are sensitive to cyanide and oxygen consumption stops in the presence of the inhibitor. Strains that were unable to induce the reporter and alternative oxidase were chosen as potential regulatory mutants for further analysis.

Using the direct selection method, 128,500 colonies were screened by the tyrosinase assay (Table 4). 1583 colonies were white, and were picked for further analysis. Of these, 146 remained white upon repeating the tyrosinase assay on individual strains, and 6 were alternative oxidase mutants when examined for inducible alternative oxidase activity on the respirometer. Because the filtration enrichment method eliminated many wild-type alternative oxidase strains, the total number of colonies screened by the tyrosinase plate assay was only 7500 (Table 4). 116 white colonies were picked and 42 remained white when tested individually. Nine of these strains were alternative oxidase mutants when tested for inducible alternative oxidase activity on the respirometer.

The total number of mutants from both selection methods was fifteen. These were named E1 through E15, and were divided between Andrea and me for further study. I worked on E4, E5, E6, E7, E9, E11, E10 and E15. I also acquired E12 after some preliminary work was conducted by Andrea. Mutants E4 and E7 were found to have an inconsistent phenotype and were not carried forward.

3.2 Characterization of Mutant Strains

3.2.1 Crosses to Generate Auxotrophic Strains

The initial mutant strains were crossed to remove secondary EMS mutations, and to introduce nutritional requirements necessary for complementation tests. I crossed the original mutant strains with strains that were wild-type for *aod-1* regulation that also carried a nutritional requirement for pantothenate or nicotinamide. Progeny requiring pantothenate or nicotinamide that were unable to induce alternative oxidase were picked for further analysis. Andrea also created a tryptophan-requiring E12 strain. All progeny selected for further work from these crosses were non-albino. These strains were chosen since mapping crosses depended on scoring conidia for colour. Since our standard lab *aod-1* mutant strain required pantothenate, a strain with a different nutritional requirement was needed to carry out complementation tests with the new mutants. I crossed the *aod-1* mutant strain with a strain requiring arginine and identified *aod-1 arg-5* progeny called AA1.

3.2.2 Screening Progeny for RIP

N. crassa has developed a mechanism to inactivate repetitive sequences by a process known as RIP (repeat-induced point mutation). It is thought that this process is a defense mechanism against transposons. During a sexual cross duplicated sequences greater than 400 bp in length and sharing more than 80% nucleotide identity undergo a series of C:G to T:A point mutations; up to 30% of the C:G pairs can be mutated (GALAGAN and SELKER 2004). In addition to sequence changes, RIPed sequences are often methylated. This process effectively inactivates the target sequence (GALAGAN and SELKER 2004).

In the EMS mutagenesis, the reporter strain and original mutant strains carried two copies of the *aod-1* promoter: the endogenous copy and an ectopic 3 kbp inserted with the reporter construct. The duplicated *aod-1* promoter sequences could therefore be RIPed and inactivated during the crosses designed to introduce nutritional requirements. If this occurred it could result in a failure to induce alternative oxidase due to the mutations in the *aod-1* promoter and I would have been unable to distinguish such *aod-1* promoter RIP mutants from the EMS mutation being studied during tests for alternative oxidase activity. Therefore, when selecting EMS regulatory mutant strains that contained nutritional requirements and were non-albino, additional tests were performed.

It was necessary to demonstrate that strains chosen for future work had lost the reporter construct during initial crosses. This would ensure that there would be no possibility of RIP inactivation of the *aod-1* promoter in subsequent crosses. Two different PCR reactions were therefore performed. The first used one primer in the tyrosinase reporter structural gene and one primer in the *aod-1* upstream sequence. This primer set would produce a product only from the integrated reporter construct, and would uncover the presence of the reporter in progeny. No band would be produced if the reporter were not present. This is shown in Figure 4A where PCR using untransformed control NCN233 gDNA as a template does not produce a band while the reporter strain T11-76 does produce a band. Progeny from the cross L2-40X763 provide an example of progeny screening. NL40-5 carries the reporter, while NL40-14 does not (Figure 4A). It was important that mutant progeny used for genetic mapping did not contain the reporter since progeny from crosses involving such strains would not need to be screened for RIP of the *aod-1* upstream sequence in subsequent crosses. The second PCR amplified the endogenous *aod-1* upstream sequence using one primer in the *aod-1* coding region and another in the upstream sequence (Figure 4B). All strains produce a band from the endogenous copy of *aod-1*.

The DNA sequence of the second PCR product was obtained and compared with the wild type sequence to look for RIP mutations. Only strains with wild-type *aod-1* promoter sequence were used for subsequent work since a lack of induction of alternative oxidase in these strains would be directly related to the selected EMS mutation. The lab has shown that elements necessary for *aod-1* induction are included in the segment of the DNA that was sequenced in these studies (M. Chae, personal communication).

3.3.3 Complementation Tests

Complementation tests in *N. crassa* are performed through the creation of forced heterokaryons (Table 5). When conidia from two strains of the same mating type, but carrying different nutritional requirements, are superimposed on minimal medium the conidia fuse to form a heterokaryon and are able to grow because of the complementary auxotrophies. Complementation of alternative oxidase mutants was tested by examining alternative oxidase activity of the heterokaryons using one of two methods. The first method was to grow the heterokaryons in a minimal medium liquid culture in the presence of chloramphenicol. Complementing mutations would result in alternative oxidase induction under these conditions, while allelic mutations should fail to complement and have only cyanide sensitive respiration. The second method used was to spot conidia on minimal medium plates containing antimycin A. In this case complementing mutations allow growth, while allelic mutations fail to grow. The mutants isolated in the screen fell into six complementation groups (Table 6), two of which (*aod-4* and *chl-2*) were characterized by Andrea (DESCHENEAU *et al.* 2005). I performed complementation tests on E5, E6, E9, E10, E11, E12 and E15. Of these, E5, E9 and E11 were found to be allelic to E1. After these complementation results, E9 and E11 were further characterized as part of Andrea's E1 analysis. E6, E10, E12, E15 were found to correspond to unique complementation groups, each represented by a single allele. Based on these data, the different complementation groups were assigned *aod* gene numbers as summarized in Table 7.

3.3.4 Respiration of the Mutants

Representative respirometer tracings for each complementation group are found in Figure 3. The reporter strain T11-76 is wild-type for alternative oxidase activity and has cyanide insensitive respiration when grown in the presence of chloramphenicol or antimycin A, but is sensitive to cyanide when grown under non-inducing conditions. In contrast, the new regulatory mutants lack cyanide insensitive respiration when grown in the presence of chloramphenicol, the same as mutants of the structural gene *aod-1* and the previously identified regulatory locus *aod-2*. Strains *aod-6* and *aod-8* are able to grow in the presence of antimycin A, but show virtually no cyanide insensitive respiration. All other mutants fail to grow in the presence of antimycin A.

3.3.5 Growth Rates

If the mutations isolated in this screen affected global mitochondrial regulation or function, it would be expected that defects in growth would result. To test this hypothesis, growth rates of the mutant strains were examined. Serial dilutions of known numbers of conidia were spotted onto plates of non-inducing medium, medium with chloramphenicol or medium with antimycin A (Figure 5). Under non-inducing conditions and in the presence of chloramphenicol all mutants grew at a rate comparable to wild-type. In the presence of antimycin A, *aod-6* and *aod-8* grew at near wild-type rates, while other mutants did not grow at all. Interestingly, when grown in liquid culture in the presence of antimycin A using similar inocula, *aod-6* grew at approximately half the rate of wild-type. The wild-type grew to 3 g fresh weight mycelium in 25 h while the *aod-6* culture required 52 h to reach the same weight. Similarly, after 48 h, a liquid culture of *aod-8* grown in the presence of antimycin A grew to 4.6 g fresh weight whereas a wild-type culture reached 8.5 g after 45 h. Since the mutants did not show different growth rates on non-inducing media or in the presence of chloramphenicol it appears that the *aod* mutations do not affect global regulatory processes under these conditions. As expected, strains unable to produce alternative oxidase could not grow in the presence of antimycin A. The reason for the difference in growth between liquid and solid medium observed in the *aod-6* and *aod-8* mutants strains is not clear.

3.3.6 Cytochrome Spectra

A mutation affecting global regulation of mitochondrial proteins would likely affect the production or accumulation of the cytochromes of the ETC. The relative cytochrome composition of mitochondria can be determined in isolated detergent-solubilized mitochondria by performing spectral analysis from 650 to 500 nm. The cytochrome content of mutants *aod-5* and *aod-7* resembled that of wild-type in non-inducing conditions or chloramphenicol. In mutants *aod-6* and *aod-8* the phenotype under non-inducing conditions also resembled wilt-type, but in the presence of chloramphenicol these strains demonstrated a slightly elevated level of cytocrhome aa_3 compared to wild-type. Members of the *aod-4* complementation group had a similar phenotype but appeared to be slightly deficient in cytochromes aa_3 and *b* (Figure 6). Only *aod-6* and *aod-8* grew in the presence of antimycin A, and those spectra generally resembled that of wild-type, but with slightly elevated cytochrome *c* levels (Figure 6). These spectra also confirmed that none of these mutants carried a chloramphenicol resistance mutation such as the strain identified by Andrea called *chl-2* (DESCHENEAU et al. 2005). When grown in chloramphenicol the cytochrome spectrum of this strain resembles that produced under non-inducing conditions, while a wild-type strain grown in chloramphenicol shows a deficiency of cytochromes aa_3 and *b*, and an increased level of cytochrome *c* (not shown). These data suggest that the *aod-4* mutation may result in global changes affecting both alternative oxidase and mitochondrial cytochromes.

3.3.7 *aod-1* Expression in Mutant Strains

Since mutants were identified by a failure to induce the tyrosinase reporter under inducing conditions, it was expected that regulatory mutants unable to induce *aod-1* transcription would be isolated by this scheme. To determine if this was actually the case, total RNA was isolated from each mutant and examined on Northern blots probed with the entire *aod-1* coding sequence (Figure 7). Usually, wild-type strains do not accumulate *aod-1* mRNA when grown in non-inducing conditions, but accumulate transcript at high levels when grown under inducing conditions (LI *et al.* 1996). The reporter strain T11-76 is somewhat unusual in that it consistently accumulates detectable amounts of *aod-1* transcript even in uninduced cultures (DESCHENEAU *et al.* 2005). This phenotype is also seen occasionally in some wild-type cultures (TANTON *et al.* 2003) but the reason for this is not clear. In contrast to wild-type, strains mutant for *aod-1* or *aod-2* do not accumulate *aod-1* mRNA under inducing or non-inducing conditions (LI *et al.* 1996). Two of my mutants, *aod-5* and *aod-7* have a similar phenotype, and do not accumulate transcript, even when grown under inducing conditions. Two mutants do not, however, behave as expected with respect to *aod-1* mRNA accumulation. Mutants *aod-6* and *aod-8* accumulate *aod-1* mRNA. *aod-6*, like the reporter strain T11-76, contains *aod-1* mRNA even under non-inducing conditions. This phenotype is consistent, having been examined in three different cultures of *aod-6*. *aod-8* has an intermediate phenotype, accumulating less mRNA under inducing conditions than wild-type or *aod-6* strains, but more than the other mutants.

3.3.8 AOD1 Protein in Mutant Strains

Isolated mitochondria from wild-type strains were examined by Western blot for the presence of AOD1 protein in cultures grown under non-inducing conditions and in cultures grown in the presence of chloramphenicol or antimycin A (Figure 7). The protein is typically absent in cultures grown under non-inducing conditions but abundant in cultures grown the presence of chloramphenicol or antimycin A (Li *et al.* 1996). This pattern holds true of the reporter strain T11-76 in spite of the fact that this strain accumulates *aod-1* transcript in uninduced cultures.

Mutations in either the structural gene *aod-1*, or in the previously known regulatory locus *aod-2*, abolished accumulation of AOD1 protein when grown in the presence of chloramphenicol. These two strains do not grow in the presence of antimycin A. Mitochondria from two of my mutants, *aod-5* and *aod-7*, do not contain AOD1 protein when grown in the presence of chloramphenicol, and as such, their phenotype is identical to the *aod-1* and *aod-2* mutant strains.

The phenotypes of the *aod-6* and *aod-8* mutants are more complex. Mitochondria from *aod-6* cultures grown in chloramphenicol consistently contain AOD1 protein, generally to half the level of a wild-type strain, although there is some variability in the amount of protein accumulated (Figure 7, Figure 8). Mitochondria from *aod-6* cultures grown in the presence of antimycin A have lower levels of protein than mitochondria from cultures induced by chloramphenicol. This is in spite of the fact that the *aod-6* mutant accumulates more *aod-1* mRNA in cultures induced by antimycin A as compared to cultures induced by chloramphenicol. Although *aod-6* cultures that are not induced accumulate *aod-1* mRNA this message does not appear to be translated into protein. The accumulation of AOD1 protein in these cultures is consistent, and was seen in four different sets of cultures of this strain.

Like *aod-6*, *aod-8* mitochondria from cultures grown under inducing conditions contain AOD1 protein, although there is less AOD1 than in wild-type mitochondria from cultures grown under similar conditions. The *aod-8* strain, unlike *aod-6*, does not accumulate mRNA under non-inducing conditions, and the mRNA and protein that accumulate in cultures grown in the presence of chloramphenicol or antimycin A appear to be present in roughly equal amounts.

3.3.9 Genetic Mapping

Genetic mapping was undertaken to localize the specific genes mutated in each complementation group to a locus on one of the seven *N. crassa* chromosomes. I crossed *aod-5* and *aod-7* strains to a mapping strain called *alcoy* for *al*bino, *co*nditional *temperature sensitive* and *y*ellow (PERKINS *et al.* 1969). This strain carries translocations involving six of the seven chromosomes in *N. crassa*. Each translocation pair carries one of the mutations, and allows mapping to two of the six chromosomes. Additional crosses are used to localize the gene to a single chromosome, and then to a region on that chromosome. *aod-6* showed weak linkage to one of the *alcoy* markers, suggesting a possible linkage to linkage group I or II (A. Todd, personal communication). *aod-5* showed linkage to the *yellow* marker indicating a location on chromosome III or VI (Table 8) Subsequent crosses showed strong linkage of *aod-5* to markers on linkage group VI (Table 8). Unfortunately the crosses indicated linkage to all three widely spaced markers on linkage group VI. *aod-7* failed to show linkage to any of the *alcoy* markers (Table 9), and was subsequently crossed to seven strains, each carrying markers for a specific chromosome. One of these crosses showed linkage to two markers, *cys-10* and *pdx-1,* on linkage group IV (Table 9).

3.4 Characterization of *aod-5*

3.4.1 Rescue of the *aod*-5 Mutation

A tiled cosmid library (KELKAR *et al.* 2001) and a genome sequence (GALAGAN *et al.* 2003) exist for *N. crassa.* Once a mutant had been genetically mapped to a specific location, various candidate cosmids from that location could be identified and used to try and rescue the mutation as determined by their ability to restore growth in the presence of antimycin A. Although *aod-5* did not map to a single location on linkage group VI, I thought that the gene could still be identified by trying to rescue the mutation with cosmids from that linkage group. Unfortunately, at the time of this experiment, only the left arm of the linkage group had been assembled, and no sequence to the right arm had been aligned with the cosmid library. In an effort to identify the gene quickly, two different approaches were taken in the lab by me and by Cheryl Nargang, a technician in the lab.

I attempted to use a sib selection approach (COFFIN *et al.* 1997) to rescue *aod-5*. I prepared pools of 48 random cosmids from the entire genomic library of approximately 3000 cosmids. By using pools of random clones from the library, I reasoned that all of linkage group VI would be included in the sibs. Each of the large pools of 48 different cosmids would be used to transform the *aod-5* strain. Once a pool of cosmids that could rescue the mutation was identified, smaller sub-pools would be isolated and used to try to rescue the mutation. Eventually, a single cosmid, or a small number of overlapping cosmids, that contained the gene mutated in the *aod-5* strain would be identified. Transformations of *N. crassa* can be done by electroporation of conidia or by transformation of sphaeroplasts. In a sib selection procedure, sphaeroplast transformation is the method of choice because a high efficiency of transformation with circular DNA molecules can be achieved. DNA must be linearized by restriction enzyme digestion for efficient transformation by electroporation, and this could disrupt the gene of interest. Unfortunately, with the original lots of sphaeroplasting enzyme we used, highly transformable sphaeroplasts could not be obtained and the sib selection procedure did not work.

As I was trying sib selection with large pools of cosmids, it was decided that Cheryl would try sphaeroplast transformation with "pools" of only two cosmids per transformation. This approach does not require a high efficiency of transformation since the complexity of the pools is low. Since I had good mapping data showing that *aod-5* was located on linkage group VI, the 128 assembled cosmids on the left arm of this chromosome were used. Cheryl eventually identified a single cosmid, pMOCosX14F7, which rescued the *aod-5* mutation as judged by its ability to restore growth to the *aod-5* mutant strain in the presence of antimycin A. Cheryl and I digested the cosmid with various enzymes and transformed the digested cosmid into the *aod-5* strain using sphaeroplasts or by electroporation. A digestion that still rescued the mutation meant that any predicted ORFs from the cosmid that were interrupted by that restriction digest could be eliminated from consideration. Digestions using *ClaI, NotI* or *XbaI* still rescued. Cheryl and I tried subcloning fragments of cosmid DNA generated by these digestions. Cheryl subcloned *XbaI* fragments of the cosmid, and isolated a clone containing a single 3.5 kbp *Xba*I fragment that was able to rescue the mutation. I subsequently cloned this fragment into pBSKII along with a gene conferring benomyl resistance to allow for selection of *N. crassa* transformants.

The 3.5 kbp fragment is predicted to contain a single ORF of 2.4 kbp by the *N. crassa* genome project. This ORF is predicted to contain one 196 bp intron near the 5' end of the gene, and to encode a predicted protein of 730 amino acids (Figure 9).

I wished to confirm that the identified ORF was the *aod-5* gene defined by our mutation. A region including the *aod-5* ORF as well as 600 bp upstream and 600 bp downstream of the ORF was obtained by PCR from the *aod-5* mutant strain. The PCR product was sequenced and compared to the wild-type sequence (database) to identify the specific mutation in this strain. A single mutation was identified in the codon for tryptophan 512 that changed the UGG to UGA producing a premature stop codon which truncated the protein by 218 amino acids (Figure 9). No other mutations or polymorphisms were identified. The AOD5 protein has strong identity to the Zn2(II)Cys6 binuclear cluster family of fungal transcription factors (see Section 3.4.5)

3.4.2 *aod-5* Expression

To analyze *aod-5* expression, total RNA was isolated from a wild-type strain and from the *aod-5* mutant strain, each grown in the presence and absence of antimycin A or chloramphenicol. Since *aod*-5 is necessary for *aod*-1 induction, it was possible that *aod-5* might also be expressed only under conditions where *aod*-1 expression was induced, such as growth in the presence of antimycin A. Northern blot analysis was performed on a blot containing 5 µg of total RNA in each lane. This blot was probed with the *aod-5* gDNA including 500 bp flanking sequence upstream and downstream of the coding region. No band was detected after one week exposure. A second Northern blot was performed on a blot containing 30 µg of total RNA. After one week there was no signal detected (not shown).

It was conceivable that under the growth conditions of the above experiment, the *aod-5* mRNA was produced transiently early in the age of the culture and rapidly degraded, particularly if *aod-5* expression was also induced by antimycin A. Wild-type liquid cultures were therefore grown overnight (approximately 16 h) under non-inducing conditions, at which point antimycin A was added to the culture. Cultures were allowed to grow in the presence of antimycin A for an additional, 15, 30, 60, 90 or 120 minutes before harvesting. To check for induction of *aod-1,* RNA was isolated from these cultures and 5 µg of total RNA was subjected to Northern analysis using the *aod-1* coding sequence as the probe. After 45 h exposure, the *aod-1* transcript was detected first in the fifteen-minute antimycin treatment culture, reached a peak in the 60-90 min cultures and then decreased in the 120 min culture (Figure 10). It seems likely that expression is decreased after 120 min because the antimycin A is no longer effectively inhibiting the ETC in the culture which contains a large amount of mycelium. In support of this notion, I have observed that wild-type cultures grown in the presence of antimycin A for 24-48 h can become very thick. When tested on an oxygen respirometer, these thick cultures no longer appear to have completely cyanide insensitive respiration. RNA from these cultures also shows decreased *aod-1* transcript levels compared to younger cultures grown in the presence of antimycin A (not shown).

Other *N. crassa* zinc cluster proteins are known the be expressed at extremely low levels (FU *et al.* 1989). Therefore 30 μg of RNA from the samples shown in Figure 10 was probed with the *aod-5* probe described above, but no signal was detected after a 45 h exposure (not shown). A further exposure of this blot was not taken because background would have obscured the region of interest. A second blot using 30 μg of RNA from these cultures and from an extended time course (see below) was probed with a slightly shorter *aod-5* probe (genomic DNA from the start codon to the stop codon) and no signal was detected after an exposure of one week (not shown).

The above results suggested that *aod-5* mRNA was present at a level too low to be detected by Northern analysis. In an attempt to detect *aod-5* transcript using a different approach, total RNA from the time course cultures was used for RT-PCR. For this experiment the antimycin A induction time course was extended with cultures induced for 180, 240, 300 and 360 min. Total RNA samples were subjected to reverse-transcription and the cDNA products amplified with *aod-5*-specific primers. This primer set was predicted to produce a band of approximately 400 bp in length from cDNA and 600 bp from genomic DNA (Figure 11). RNA samples from wild type and mutant cultures grown under non-inducing conditions or in the presence of chloramphenicol were also tested. The RT-PCR showed that *aod-5* was expressed in wild-type uninduced cultures, as well as in cultures induced with either chloramphenicol or antimycin A. Sequence of the PCR product was obtained and the results confirmed that the 400 bp cDNA product lacked the predicted intron. The *aod-5* transcript was also detected in induced and uninduced cultures of the *aod-5* mutant. These data show that *aod-5* is expressed in both inducing and non-inducing conditions, though conclusions about the levels of transcript present under the various conditions cannot be drawn.

3.4.3 Comparison of *N. crassa* AOD5 to Protein Database Sequences

When the protein sequence of AOD5 was compared to the sequences of other proteins in the EXPASY/UniProtKB complete databases using BLAST (basic local alignment search tool) (ALTSCHUL *et al.* 1997), several homologues were identified (Table10) (Figure 12). This may be due to the large number of zinc cluster proteins encoded in fungal genomes (SCHJERLING and HOLMBERG 1996; TODD and ANDRIANOPOULOS 1997). Four proteins were very closely related to AOD5. First was the hypothetical protein FG0826.1 from *Gibberella zeae*. This 665 amino acid protein had 317 (317/665 = 48%) identical amino acids and 407 (61%) similar amino acids. The second protein was putative Cys6 zinc cluster domain protein Afu2g05830 from *Aspergillus fumigatus*. This 739 amino acid protein had 310 (out of 739 or 38%) identical amino acids and 374 (50%) similar amino acids. Third was the 688 amino acid predicted protein AO090001000703 from *Aspergillus oryzae*. This protein had 288 (41%) identical amino acids and 374 (54%) similar amino acids. The final homologue with high identity was the 702 amino acid hypothetical protein AN7468.2 from

Aspergillus nidulans. This protein had 307 identical residues (273/702 38%) and 374 (53%) similar residues. An alignment of the *N. crassa, G. zeae* and *A. nidulans* protein is shown in Figure 12.

3.4.4 *Aspergillus nidulans aod-5*

It was of interest to determine if a homologue to *N. crassa aod-5* could rescue the defect in the *N. crassa* mutant strain. *A. nidulans* gDNA was available in the lab (from M. Chae) to test this hypothesis. Primers were designed to amplify a region of DNA containing the predicted coding sequence and 500 bp of flanking DNA from *A. nidulans* genomic DNA (Figure 13). This fragment, along with the gene conferring benomyl resistance, were cloned into pBSKII to form plasmid pBNAsp5. This plasmid was transformed into conidia of the *N. crassa aod-5* mutant strain by electroporation, and the conidia were plated on medium containing benomyl or antimycin. Colonies formed on medium containing benomyl, confirming successful integration of the plasmid while colonies that formed on medium containing antimycin A indicated rescue of the *aod-5* mutation and restoration of *aod-1* activity. Similar numbers of colonies were seen with a transformation using the *N. crassa aod*-5 gene (plasmid pBNA5-2) while no colonies were observed on either medium in the “no DNA” negative control (Figure 14).

3.4.5 AOD5 Protein Domains

The AOD5 protein is predicted to have a number of conserved protein domains (Figure 12). The most obvious of these is the Zn(II)2Cys6 binuclear cluster near the N-terminus of the protein. This motif consists of six cysteine residues with a conserved spacing. In the folded protein these residues coordinately bind two zinc ions (SCHJERLING and HOLMBERG 1996; TODD and ANDRIANOPOULOS 1997). This motif is thought to bind DNA and activate transcription of target genes. The consensus sequence for the zinc cluster domain is $CX_2CX_6CX_{5-16}CX_2CX_{6-8}C$ (LIU and MARZLUF 2004; TODD and ANDRIANOPOULOS 1997) while the sequence for AOD5 is $CX_2CX_6CX_5CX_2CX_8C$. A predicted nuclear localization signal, consistent with the role of this protein as a transcription factor, is also present near the N-terminus. As in another *N. crassa* zinc cluster protein called *fluffy*, the intron in *aod-5* interrupts the DNA binding domain (BAILEY and EBBOLE 1998). Following the zinc cluster is a small basic region whose function is unknown.

Downstream of the zinc cluster is the so-called "middle homology region" (MHR). This region has no clearly defined function, but is conserved in a number of fungal zinc cluster proteins. It has been hypothesized that this region may play a role in defining target specificity for the transcription factor (SCHJERLING and HOLMBERG 1996).

A large region located in the second half of the protein contains a PAS domain, and is predicted to form a conserved PAS fold (PONTING AND ARAVIND 1997). This tertiary structure often plays a role in transcription factor activation and signal transduction. The PAS domain can bind signal transducing ligands such as heme groups (GILLES-GONZALEZ and GONZALEZ 2004), or can bind other PAS domains in other proteins, thereby forming homo- or heterodimers. Generally only a few residues of the primary sequence of the PAS domain are well conserved, but the three dimensional structure of the fold is well conserved (HEFTI *et al.* 2004; PONTING and ARAVIND 1997).

3.4.6 Site-directed Mutagenesis

To examine the role of the predicted domains in AOD5 function, a series of mutations was made that affected specific conserved residues in these regions. An alignment (Figure 12) of AOD5 homologue protein sequences with the highest identity was used to identify well-conserved residues in related proteins. In addition, a review of PAS domains (PONTING and ARAVIND 1997) containing sequences from a wide range of species in all kingdoms was used to identify residues that were widely conserved in this domain, even in unrelated, highly divergent species.

Mutations were created by a PCR-based site-directed mutagenesis procedure. A plasmid containing *aod-5* genomic sequence, and carrying a benomyl resistance selectable marker, pBNA5-2 was used as the template for mutagenesis. A total of six separate changes were made. The first mutation (C91S) changed one cysteine within the zinc cluster to a serine. This was expected to disrupt binding of one of the zinc ions and eliminate the function of the protein (TODD and ANDRIANOPOULOS 1997). The second mutation (ΔR474) deleted the codon for arginine 474 just upstream of the predicted PAS domain. This mutation might disrupt the PAS fold and eliminate the function of the protein. The third mutation (ΔS488) deleted the codon for serine 488 of the PAS domain. This mutation might disrupt the PAS fold and eliminate the function of the protein. After sequencing the mutant plasmid, it was discovered that due to an error in the mutagenic primer sequence the gene also contained a single base pair deletion, leading to a frame-shift mutation, in addition to carrying a deletion of the targeted codon. The name of the mutation was changed to ΔS488^{FS} and was used as a control that should not be functional. The fourth mutation (ΔG497) deleted the codon for glycine 497 of the PAS domain. This mutation might disrupt the PAS fold and eliminate the function of the protein. The fifth mutation was a substitution that changed glycine 497 to alanine. G497 was a conserved residue in the PAS domain, and was expected to be important, but a change to an alanine might or might not be expected to disrupt the PAS fold. The final mutation (ΔE505) deleted the codon for glutamic acid 505 of the PAS domain. This mutation might disrupt the PAS fold and eliminate the function of the protein.

After mutagenesis, sequence was obtained for the entire *aod-5* coding region in each mutant plasmid to confirm the introduction of the desired mutation, and the absence of any additional mutations. These plasmids were transformed into the *aod-5* strain by electroporation and the transformed conidia were plated on medium containing antimycin A to determine if the mutated allele could rescue *aod-5* function. Transformed conidia were also plated on medium containing benomyl as a positive control for electroporation (Figure 15). No colonies appeared on either medium in the "no DNA" negative control. Transformation with pBNA5-2, the plasmid containing benomyl resistance and the wild-type *aod-5* sequence, resulted in colonies on both media. The site-directed mutant plasmids generated many colonies on plates containing benomyl, but no colonies were seen on plates containing antimycin A. Thus, each of the mutations made in this study prevented zinc binding (C91S) or prevented proper protein folding of the AOD5 protein (ΔR474 ΔS488^{FS} ΔG497 G497A ΔE505).

3.4.7 Attempts to Produce Antibodies Against AOD5

Two attempts were made at generating an antibody to AOD5, each time using two regions of the protein as antigens. The first region was 96 amino acids from the middle of the protein while the second was the 114 amino acids of the C-terminus. These regions were chosen because neither showed homology to other *N. crassa* proteins, and would therefore be more likely to produce a specific antibody. The portions of the gene encoding these residues were cloned into expression vector pQE-40 where the amino acids would be fused to a his-tagged DHFR to allow for purification. The plasmids were transformed into *E. coli* cells where expression of the fusion protein was induced. Purified fusion proteins were isolated by NiNTA agarose column chromatography and were injected into rabbits and guinea pigs. After five injections, serum from test bleeds was used against purified DHFR, the purified fusion proteins, nuclear protein extracts, and inclusion bodies containing AOD5 expressed in *E. coli* (from M. Chae). Antibodies reacted with the fusion protein, but also reacted with purified DHFR and an unknown high molecular weight protein in the purified fusion protein sample. Neither antigen in any animal produced an antibody that recognized a band of the correct size against any of the substrates, except the fusion protein. Work in this direction was therefore suspended.

3.4.8 Creation of a His-tagged Version of AOD5

To facilitate protein detection and purification, a six-histidine residue tag was inserted into the AOD5 sequence. In one construct the tag was inserted immediately after the ATG by PCR mutagenesis (plasmid pHisaod-5N). A summer student, Colin Lin, created the second construct by inserting the tag immediately preceding the stop codon (plasmid pHisaod-5C). Both constructs were transformed into the *aod-5* mutant strain by electroporation and conidia were plated onto medium containing antimycin A. Conidia transformed with either tagged construct, but not untransformed conidia, were able to grow in the presence of antimycin A (Figure 16 N-terminal tag, C-terminal tag results not shown). This indicated that the his-tagged forms of the protein were able to function.

In an attempt to purify AOD5 protein directly from *N. crassa* cells, nuclei were isolated from an untransformed wild type strain and strains carrying the N-terminal His-tagged version of AOD5. Nuclear protein extract was subjected to SDS-PAGE, followed by a Western blot. An antibody against histone H3 (from M. Schultz) was used to confirm that the nuclear isolation procedure successfully isolated nuclei (Figure 17). When probed with a penta-his antibody multiple, identical bands of various sizes were seen in the wild-type and His tag strain nuclear proteins (not shown). To reduce this background, nuclear protein extract was applied to a Ni-NTA column. Purified elution fractions were then analyzed by Western blot and several bands of various sizes were detected in the flow-through. An intense band greater than 82 kDa was detected by the penta-his antibody in the eluate. Unfortunately, identical bands were present in both wild-type and his-tagged purified nuclear protein eluate and the size was greater than the 80 kDA expected for AOD5 (Figure 18). This experiment was repeated with the C-terminal his tag strain, but again no difference was seen between wild-type nuclei and nuclei from the his tag strain (not shown). Cytosol from the N-terminal his-tag strain was also screened in case the protein was translocated to the nucleus under specific conditions. No band of the correct size was detected in the cytosol (Figure 18). Given the fact that *aod-5* mRNA cannot be detected by Northern blot analysis, it seems likely that the AOD5 protein is not abundant.

3.5 Characterization of *aod-6* and AOD1 Protein Assembly

3.5.1 *aod-1* mRNA in the *aod-6* Mutant

Like the reporter strain T11-76, the *aod-6* strain chosen for further work (EL62-2) consistently accumulates *aod-1* mRNA under non-inducing conditions. When grown in the presence of chloramphenicol mRNA is also present, although somewhat less than in wild-type cultures grown in chloramphenicol. Growth in the presence of antimycin A leads to accumulation of *aod-1* mRNA to levels comparable to wild-type (see Section 3.3.6) (Figure 7). This strain consistently accumulates more mRNA when induced by antimycin A than when induced by chloramphenicol.

3.5.2 AOD1 Protein in the *aod-6* Mutant

Although AOD1 protein is present in *aod-6* mitochondria grown under inducing conditions, there is less protein than in wild-type strains (Figure 7). This was surprising given the amounts of *aod-1* mRNA observed, particularly in cultures grown in the presence of antimycin A. Mitochondria from this *aod-6* strain consistently have more AOD1 protein when grown in the presence of chloramphenicol than when grown in the presence of antimycin A, although there can be some variability in the amount of AOD1 protein accumulated in this strain (Figure 7, Figure 8). In addition, *aod-6* shows virtually no cyanide-insensitive respiration when grown in the presence of chloramphenicol or antimycin A (Figure 3, Figure 8).

3.5.3 Reporter Activity in the *aod-6* Mutant

The presence of *aod-1* mRNA and a reduced amount of AOD1 protein in mitochondria of the *aod-6* strain is difficult to reconcile with the lack of reporter activity in this strain when grown under inducing conditions that were used to select the mutant. The reduced protein levels suggest that this strain carries a mutation that affects translation of *aod-1* transcript, however in the reporter the tyrosinase structural gene is governed by the *aod-1* promoter so that a defect in alternative oxidase translation should have no impact on activation of the promoter. Thus a translation defect should not have been isolated by the mutagenesis screen. Repeated testing of the original *aod-6* mutant isolate confirmed that reporter expression was reduced in this strain (Figure 2). One possible explanation for isolating a strain with a putative translational defect is that the *aod-6* strain actually contains two mutations: one affects AOD1 protein production, and the other is a mutation in the reporter itself.

To examine this possibility, sequence was obtained from a PCR product of the integrated reporter fusion containing *aod-1* upstream region and tyrosinase coding regions of the reporter. No changes were found in the tyrosinase gene. When the sequence of the *aod-1* upstream region of the reporter from the *aod-6* strain was compared to wild-type sequence, a single mutation was observed. The C at position –379 (relative to the *aod-1* translational start site) was changed to a T. This mutation is outside the minimum promoter region necessary for *aod-1* induction (TANTON *et al.* 2003), and is not in any predicted motif. It therefore seems unlikely that this mutation is responsible for the lack of reporter function in this strain.

Sequence was also obtained from the endogenous *aod-1* gene in this strain to eliminate the possibility that a mutation in the endogenous gene was responsible for reduced alternative oxidase protein production or activity in the *aod-6* strain. The sequence obtained was wild-type, both in the upstream region and coding sequence.

3.5.4 Subcellular Localization of AOD1 in the *aod-6* Mutant

Because mitochondria from the *aod-6* strain showed lower levels of AOD1 protein, but normal levels of *aod-1* transcription, it was possible that the protein was being produced, but not being properly imported into mitochondria. Therefore the cellular localization of AOD1 was examined in the *aod-6* mutant strain. Mitochondria and a post-mitochondrial cytoplasmic supernatant were isolated and examined by Western blot. In both wild-type and *aod-6* cells there was no AOD1 protein under non-inducing conditions. In the wild-type cultures grown in the presence of chloramphenicol AOD1 was localized to mitochondria (Figure 19). In *aod-6* cultures grown in chloramphenicol there was a lower level of AOD1 present, but its pattern of localization was identical to wild-type (Figure 19). These data indicate that there is no mis-localization of AOD1 in the *aod-6* strain.

3.5.5 Mitochondrial Localization of AOD1 in the *aod-6* Mutant

The AOD1 protein is synthesized and localized to mitochondria in the *aod-6* strain, and therefore the almost total lack of cyanide insensitive respiration (Figure 3) in the strain is unexpected. One possible explanation is that the AOD1 protein is reaching the mitochondria, but is not being properly sorted or assembled.

Mitochondria from a wild-type strain (NCN233), the parents of the *aod-6* strain (T11-76 and 7262) and the *aod-6* strain (EL62-2) were subjected to sonication to determine if there was a difference in release of AOD1 from association from the MIM. Mitochondria were subjected to 1, 2, 4, 6, or 12 bursts of sonication of 10s each, followed by ultracentrifugation to separate the pellet and the supernatant. Control mitochondria were not sonicated, but subjected to centrifugation. As expected, these control mitochondria showed AOD1 only in the pellet (Figure 20). In the wild-type strain and 7262 increased sonication resulted in a larger proportion of AOD1 found in the supernatant until at 6 or 12 bursts, the protein was found equally in both fractions. In the *aod-6* strain and T11-76, no AOD1 was seen in the supernatant until 6 or 12 bursts of sonication, when some AOD1 began to appear in the supernatant. These data suggest that mitochondria from T11-76 and the *aod-6* strain are more resistant to sonication compared to a wild-type strain and 72-62, since more sonication is required to release AOD1 in the *aod-6* strain and T11-76. Two control proteins were used: the matrix protein Hsp70 and the integral MIM protein Tim17. Hsp70 was found in both fractions at all sonication times tested, while Tim17 was found consistently in the pellet. These data suggest that mitochondria were not being totally disrupted by this experiment.

3.5.6 Growth of the *aod-6* Strain at Different Temperatures

To test whether the *aod-6* mutation might affect the ability of *N. crassa* to grow at different temperatures known numbers of conidia from a wild-type strain, the *aod-6* strain, and parental strains T11-76 and 7262 were spotted onto non-inducing medium and medium containing chloramphenicol or antimycin A. Plates were incubated at 10°C, 30°C and 37°C to test if temperature affected the ability of *aod-6* to grow on solid medium. No large differences were seen between the four strains when grown at any temperature (Figure 21).

3.6 Characterization of *aod-8*

Because the *aod-8* mutant strain contained a small amount of transcript and protein (Figure 7), but did not activate the reporter (Figure 3) it was possible that this strain also carried a reporter mutation. When sequence of a PCR product of the reporter construct from the *aod-8* strain was compared to wild-type sequence, a single mutation was observed in the promoter region of the reporter. The T at position –624 (relative to the *aod-1* translational start site) was changed to a C. Like the mutation in the reporter of *aod*-6, this mutation lies outside the minimum promoter region necessary for *aod-1* induction (TANTON *et al.* 2003), and is not in any predicted motif. It seems doubtful that this mutation is responsible for the lack of reporter function in this strain. It is conceivable that there may be another mutation elsewhere that is affecting reporter function. Sequence from the endogenous *aod-1* promoter and coding region were also compared to wild-type sequence and found to contain no changes.

3.7 Import of AOD1 into Mitochondria

To date, nothing is known about how AOD1 is assembled into its functional conformation in the matrix, associated with the MIM.

A protein of the mitochondrial export machinery, OXA1, has been shown to insert some proteins from the matrix into the MIM (NARGANG *et al.* 2002). To test the hypothesis that OXA1 may play a role in AOD1 assembly, an *in vitro* import assay of AOD1 into an OXA1 deficient strain was performed. Because OXA1 is an essential protein, the deficient strain had been created by sheltered RIP, where one nucleus bears a wild-type copy of *oxa-1* and a nutritional marker, while the second nucleus carries the ripped *oxa-1* and a different selectable marker, in this case resistance to the translational inhibitor cycloheximide (Figure 22). When this strain is grown in the presence of cycloheximide, the nucleus with the RIPped *oxa-1* will predominate, lowering the levels of OXA1 protein present in the culture.

I first tested to see if AOD1 protein imported into isolated wild-type mitochondria would give the same pattern of fractionation following sodium carbonate (pH 11.5) extraction as AOD1 imported normally *in vivo* following sodium carbonate (pH 11.5) extraction. Sodium carbonate (pH 11.5) treatment extracts protein from the mitochondrial membranes. An integral membrane protein would be expected to be resistant to carbonate extraction and remain associated with the membranes after centrifugation. In contrast, a soluble protein or a protein only peripherally associated with the membrane would be expected to be in the supernatant after carbonate treatment and centrifugation. A structurally related protein, the DMQ hydroxylase, has been subjected to carbonate extraction previously. In yeast it was not removed from the membrane in sodium carbonate (pH 11.5) (JONASSEN *et al.* 1998), while in mouse the same protein was removed from the MIM by sodium carbonate treatment (pH 11.5) (JIANG *et al.* 2001). In mitochondria from a control *N. crassa* strain (NCN233) AOD1 was partially carbonate extractable, being detectable in both the membrane pellet and the supernatant in approximately equal amounts (Figure 20). There was no difference between mitochondria from chloramphenicol or antimycin A induced cultures (not shown).

Import was conducted with radiolabelled AOD1, for 1, 8, 20, and 60 min, after which the mitochondria were subjected to carbonate extraction, to examine the localization of AOD1 and control proteins. As seen in the *in vivo* carbonate extraction experiment (Figure 23) a portion of AOD1 protein was carbonate extractable, while an equal portion was not extractable (Figure 24).

To determine if less AOD1 would be imported into the membrane in OXA1-deficient mitochondria, import of radiolabelled AOD1, OXA1, F1β and Tim23 precursor proteins into oxa-1^{RIP} and parental strain HV mitochondria (isolated from cells grown in the presence of cycloheximide for 48 and 24 h respectively) was performed in duplicate for 8 and 20 min. One sample was subjected to SDS-PAGE (Figure 24A) to show that all precursors would be efficiently imported into mitochondria while the second sample was subjected to sodium carbonate (pH 11.5) extraction. Proteins AOD1, F1β and OXA1 *in vitro* synthesized precursor proteins contain targeting sequences which are cleaved upon import into mitochondria. The precursor proteins in the lysate are therefore larger than the mature proteins found inside the mitochondria after import has taken place. Proteins Tim23 and OXA1 are integral membrane proteins of the MIM and as expected were found in the membranous pellet but were absent from the supernatant. Protein F1β is a peripheral membrane protein, and as expected was solubilized by sodium carbonate (pH 11.5) treatment. In both strains imported AOD1 protein was present in both the pellet and supernatant following the extraction (Figure 25B). This indicates that assembly of AOD1 at the MIM does not depend on OXA1. In contrast, less OXA1 was found in the membrane fraction in the OXA1-deficient strain. This was expected since OXA1 import is dependent on OXA1 protein.

Table 4.
Mutants isolated from EMS mutagenesis.

EMS Method	Colonies Screened[1]	White Colonies Picked	Characterized by Respiration	Mutant Strains Resulting
Direct Selection Trial 1	19600	104	18	
Direct Selection Trial 2	39300	211	16	E1
Direct Selection Trial 3	37300	353	40	E2
Direct Selection Trial 4	4400	48	9	E3, E4
Direct Selection Trial 5	21900	432	27	E7
Direct Selection Trial 6	5100	402	29	E15
Direct Selection Trial 7	900	33	7	
Filtration Enrichment Trial 1	2×10^8, 600[2]	20	5	E5, E6
Filtration Enrichment Trial 2	8×10^8, 4900	71	24	E8, E9, E10, E11 E12, E13, E14
Filtration Enrichment Trial 3	8×10^8, 2000	25	13	
Total Method 1	128 500	1583	146	6
Total Method 2	1.8×10^9, 7500	116	42	9

[1] The number of colonies per plate was counted for 25 plates and the average was used to extrapolate the total colonies screened.

[2] The first number indicates the total number of conidia treated with EMS and inoculated into antimycin A containing liquid medium, while the second number indicates the number of colonies that grew when the remaining conidia were plated after enrichment.

A similar table appears in the thesis of Andrea Todd (Descheneau).

Table 5.

Heterokaryotic strains created for complementation analysis.

Heterokaryon	Combination	Result
PL16-63 + 7064	E5 + *aod-2*	complements
PL16-56 + NL37-98	E5 + E9	fails to complement
PL16-56 + NL61-130	E5 + E11	fails to complement
PL16-56 + NN64-29	E5 + E15	complements
PL1377-51 + AA1-12	E6 + *aod-1*	complements
PL1377-24 + 7064	E6 + *aod-2*	complements
PL1377-51 + EN294-46	E6 + E2	complements
PL1377-66 + EN294-46	E6 + E2	complements
PL1377-24 + NL40-14	E6 + E10	complements
PL1377-51 + NL40-5	E6 + E10	complements
PL1377-66 + NL40-5	E6 + E10	complements
PL1377-51 + NL61-130	E6 + E11	complements
PL1377-66 + NL61-130	E6 + E11	complements
PL1377-51 + EL62-2	E6 + E12	complements
PL1377-66 + EL62-2	E6 + E12	complements
PL1377-24 + NN64-35	E6 + E15	complements
PL1377-51 + NN64-33	E6 + E15	complements
PL1377-66 + NN64-33	E6 + E15	complements
NL37-98 + 7207	E9 + *aod-1*	complements
PL37-20 + 7064	E9 + *aod-2*	complements
NL37-98 + EN195-34	E9 + E1	fails to complement
PL37-1 + EN294-46	E9 + E2	complements
PL37-1 + EN14-34	E9 + E3	complements
PL37-1 + EL25-8	E9 + E8	fails to complement
NL37-98 + EL25-8	E9 + E8	fails to complement
NL37-98 + PL61-54	E9 + E11	fails to complement
NL37-31 + PL61-54	E9 + E11	fails to complement
PL37-1 + EL62-25	E9 + E12	complements
PL37-1 + EL64-47	E9 + E13	fails to complement
NL37-98 + EL64-47	E9 + E13	fails to complement
NL37-98 + PN64-87	E9 + E15	complements
NL37-98 + PN64-91	E9 + E15	complements
PL40-23 + AA1-37	E10 + *aod-1*	complements
NL40-33 + 7207	E10 + *aod-1*	complements
NL40-46 + 7207	E10 + *aod-1*	complements
NL40-5 + 7207	E10 + *aod-1*	complements
NL40-30 + 7207	E10 + *aod-1*	complements
PL40-33 + 7064	E10 + *aod-2*	complements
PL40-35 + EN294-46	E10 + E2	complements
PL40-23 + NL37-31	E10 + E9	complements

PL40-35 + NL37-31	E10 + E9	complements
PL40-33 + NL61-129	E10 + E11	complements
NL40-5 + EL62-2	E10 + E12	complements
NL40-30 + EL62-2	E10 + E12	complements
NL40-5 + EL62-25	E10 + E12	complements
NL40-33 + EL62-2	E10 + E12	complements
NL40-46 + EL62-2	E10 + E12	complements
PL40-10 + EL62-2	E10 + E12	complements
NL40-24 + EL62-2	E10 + E12	complements
PL40-33+ EL62-25	E10 + E12	complements
NL40-30 + EL62-25	E10 + E12	complements
NL40-24 + EL62-25	E10 + E12	complements
NL40-46 + EL62-25	E10 + E12	complements
NL40-10 + EL62-25	E10 + E12	complements
PL40-12 + NN64-29	E10 + E15	complements
PL40-35 + NN64-29	E10 + E15	complements
NL40-32 + PN64-69	E10 + E15	complements
NL40-14 + PN64-69	E10 + E15	complements
NL61-130 + 7207	E11 + *aod-1*	complements
PL61-5 + 7064	E11 + *aod-2*	complements
NL61-19 + EN195-26	E11 + E1	fails to complement
NL61-119 + EN195-34	E11 + E1	fails to complement
NL61-128 + EN195-34	E11 + E1	fails to complement
NL61-130 + EN195-34	E11 + E1	fails to complement
PL61-54 + EN294-46	E11 + E2	complements
NL61-127 + EN294-46	E11 + E2	complements
PL61-54 + EN14-34	E11 + E3	complements
NL61-127 + EL25-8	E11 + E8	fails to complement
NL61-128 + EL25-8	E11 + E8	fails to complement
NL61-130 + EL25-8	E11 + E8	fails to complement
NL61-19 + PL37-20	E11 + E9	fails to complement
NL61-19 + PL37-38	E11 + E9	fails to complement
NL61-128 + PL37-1	E11 + E9	fails to complement
NL61-127 + PL37-28	E11 + E9	fails to complement
NL61-128 + PL37-20	E11 + E9	fails to complement
NL61-129 + PL37-38	E11 + E9	fails to complement
NL61-119 + PL37-28	E11 + E9	fails to complement
NL61-119 + PL37-1	E11 + E9	fails to complement
NL61-127 + PL37-28	E11 + E9	fails to complement
NL61-130 + PL37-28	E11 + E9	fails to complement
NL61-130 + PL37-1	E11 + E9	fails to complement
NL61-128 + PL37-28	E11 + E9	fails to complement
NL61-127 + EL62-25	E11 + E12	complements
NL61-128 + EL62-25	E11 + E12	complements
NL61-130 + EL62-25	E11 + E12	complements
NL61-127 + EL64-47	E11 + E13	fails to complement
NL61-128 + EL64-47	E11 + E13	fails to complement

NL61-130 + EL64-47	E11 + E13	fails to complement
NL61-127 + PN64-91	E11 + E15	complements
NL61-127 + PN64-87	E11 + E15	complements
NL61-119 + PN64-87	E11 + E15	complements
NL61-130 + PN64-91	E11 + E15	complements
NN64-29 + 7207	E15 + *aod-1*	complements
NN64-14 + AA1-15	E15 + *aod-1*	complements
NN64-33 + AA1-27	E15 + *aod-1*	complements
PN64-69 + 7064	E15 + *aod-2*	complements
NN64-35 + EN195-26	E15 + E1	complements
PN64-2 + EN294-46	E15 + E2	complements
PN64-79 + EN294-46	E15 + E2	complements
PN64-87 + EN294-46	E15 + E2	complements
PN64-91 + EN294-46	E15 + E2	complements
PN64-2 + EN14-34	E15 + E3	complements
PN64-79 + EN14-34	E15 + E3	complements
PN64-87 + EN14-34	E15 + E3	complements
PN64-91 + EN14-34	E15 + E3	complements
NN64-29 + PL61-54	E15 + E5	complements
NN64-1 + PL61-54	E15 + E5	complements
PN64-87 + EL25-8	E15 + E8	complements
PN64-91 + EL25-8	E15 + E8	complements
NN64-29 + PL37-28	E15 + E9	complements
NN64-35 + PL37-20	E15 + E9	complements
PN64-87 + EL62-25	E15 + E12	complements
PN64-91 + EL62-25	E15 + E12	complements
PN64-87 + EL64-47	E15 + E13	complements

Table 6.
Complementation analysis using heterokaryotic strains.

Mutant Strain	Mutant Strains												
	E1	E2	E3	E5	E6	E8	E9	E10	E11	E12	E13	E14	E15
aod-1	√[1,2]	√	√	ND[3]	√	√	√	√	√	√	√	√	√
aod-2	√	√	X	√	√	√	√	√	√	√	√	√	√
E1		√	√	ND	ND	X	X	ND	X	√	X	X	√
E2			√	ND	√	√	√	√	√	√	√	√	√
E3				ND	ND	√	√	ND	√	√	√	√	√
E5					ND	ND	X	ND	X	ND	ND	ND	√
E6						ND	ND	√	√	√	ND	ND	√
E8							X	ND	X	√	X	X	√
E9								√	X	√	X	X	√
E10									√	√	ND	ND	√
E11										√	X	X	√
E12											√	√	√
E13												X	√
E14													ND

[1] Complementation is indicated by a check mark, non-complementation by an X.

[2] The lightly shaded areas were assayed by another graduate student (A. Todd née Descheneau)

[3] ND indicates no data, since once it was discovered that mutations E1, E5, E8, E9, E11, E13, and E14 were allelic, and mutations *aod-2* and E3 were allelic, not all combinations with each of these mutations were analyzed.

A similar table appears in the thesis of Andrea Todd (Descheneau).

Table 7.
Assignment of *aod* gene designations to new alternative oxidase regulatory mutant complementation groups.

EMS Mutant Strain	*aod* Gene Designation
E1, E5, E8, E9, E11, E13, E14	*aod-4*
E10	*aod-5*
E12	*aod-6*
E15	*aod-7*
E6	*aod-8*

A similar table appears in the thesis of Andrea Todd (Descheneau).

Table 8
Mapping of the *aod-5* Mutation

Mated Strains	Marker % Recombination and χ^2 Analysis	Marker % Recombination and χ^2 Analysis	Marker % Recombinatio and χ^2 Analysis
PL40-23 (*aod-5, A*) X 998 (*alcoy, a*)	*al-1* 53 $\chi^2 = 0.222$ $p < 0.75$	*cot-1* 51 $\chi^2 = 0.0139$ $p < 0.90$	*ylo-1* 4 $\chi^2 = 66.125$ $p < 0.001$
PL40-23 (*aod-5, A*) X 1208 (*trp-1, ylo, a*)	*trp-1* 49 $\chi^2 = 0.25$ $p < 0.75$	*ylo-1* 37 $\chi^2 = 3.36$ $p < 0.10$	
PL40-23 (*aod-5, A*) X 4120 (*acr-2, trp-1, dow, a*)	*trp-1* 50 $\chi^2 = 0$	*acr-2* 45 $\chi^2 = 0.421$ $p < 0.75$	*dow* 43 $\chi^2 = 0.658$ $p < 0.50$
PL40-23 (*aod-5, A*) X 7564 (nit-6, ylo-1, un-23)	*nit-6* 10 $\chi^2 = 30.562$ $p < 0.001$	*ylo-1* 13 $\chi^2 = 25.985$ $p < 0.001$	*un-23* 16 $\chi^2 = 21.778$ $p < 0.001$

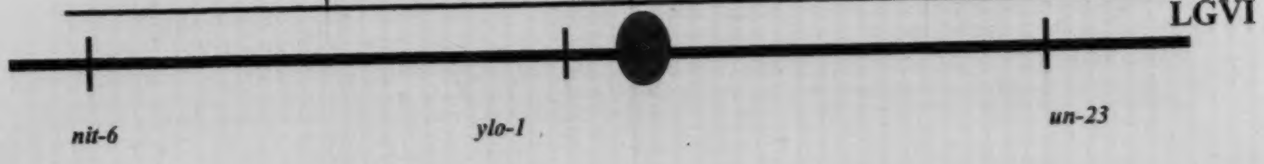


Table 9
Mapping of the *aod-7* Mutation

Mated Strains	Marker % Recombination and χ^2 Analysis	Marker % Recombination and χ^2 Analysis	Marker % Recombination and χ^2 Analysis
NN64-1 (*aod-7, A*) X 998 (*alcoy, a*)	*al-1* 43 $\chi^2 = 0.735$ $p < 0.50$	*cot-1* 66 $\chi^2 = 3.559$ $p < 0.10$	*ylo-1* 63 $\chi^2 = 1.000$ $p < 0.50$
PN64-69 X 6563 (*ro-7, arg-5, rip-1, A*)	*ro-7* 49 $\chi^2 = 0.005$ $p < 0.95$	*arg-5* 42 $\chi^2 = 1.184$ $p < 0.30$	*rip-1* 45 $\chi^2 = 0.426$ $p < 0.75$
PN64-69 X 4141 (*nic-3, met-7, arg-10, A*)	*nic-3* 52 $\chi^2 = 0.047$ $p < 0.90$	*met-7* 49 $\chi^2 = 0.005$ $p < 0.95$	*arg-10* 54 $\chi^2 = 0.258$ $p < 0.75$
PN64-69 X 4121 (*cys-10, pdx-1, pan-1, A*)	*cys-10* 33 $\chi^2 = 8.113$ $p < 0.005$	*pdx-1* 18 $\chi^2 = 29.803$ $p < 0.001$	

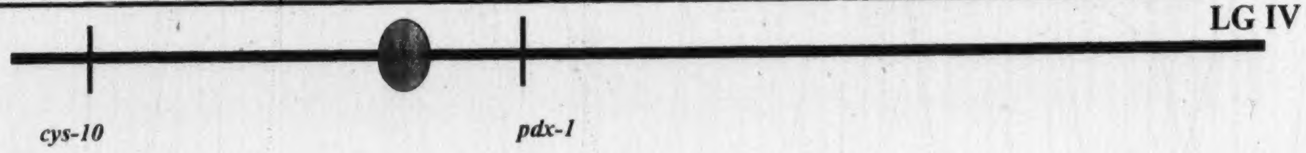


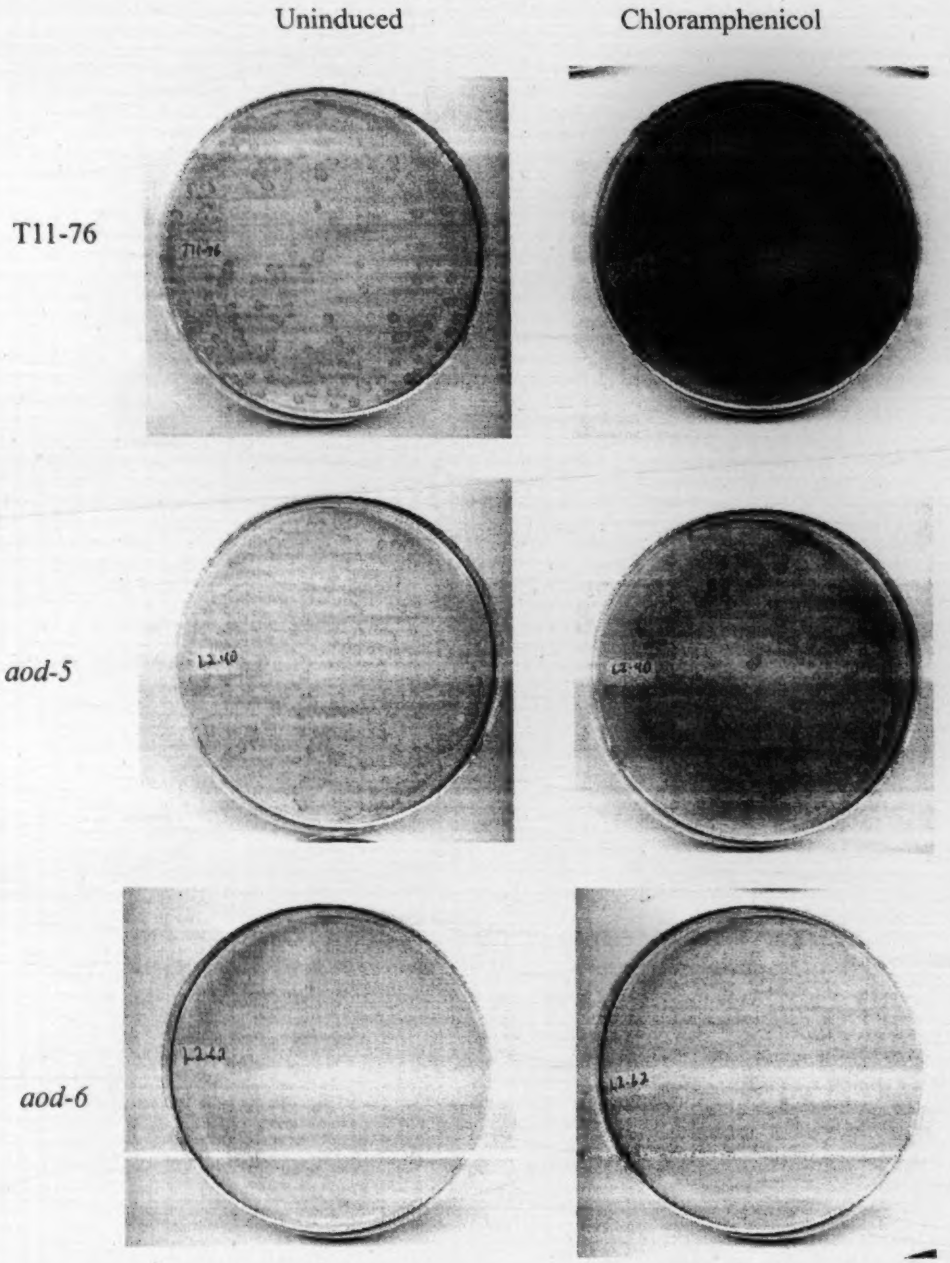
Table 10.

BLAST Results of *N. crassa* AOD5

Length	Protein	Species	Score	E-value	Identical	Similar
665	Hypothetical protein [FG08626.1]	*Gibberella zeae*	Score = 537 bits (1383)	Expect = e-151	317	407
739	C6 finger domain protein, putative [Afu2g05830]	*Aspergillus fumigatus*	Score = 443 bits (1139)	Expect = e-122	281	374
688	Predicted protein [AO090001000703]	*Aspergillus oryzae*	Score = 429 bits (1104)	Expect = e-118	288	374
732	Hypothetical protein [AN7468.2]	*Aspergillus nidulans*	Score = 420 bits (1079)	Expect = e-115	273	374
559	Similar to *S. cerevisiae* YBR239c	*Yarrowia lipolytica*	Score = 157 bits (398)	Expect = 9e-37	137	207
520	Hypothetical protein [CaO19.2423]	*Candida albicans*	Score = 150 bits (380)	Expect = 1e-34	132	195
571	Similar to CA3041|IPF7303 *C. albicans*	*Debaryomyces hansenii*	Score = 142 bits (358)	Expect = 4e-32	136	190
1050	Hypothetical protein [UM01925.1]	*Ustilago maydis*	Score = 141 bits (355)	Expect = 9e-32	130	204
529	Putative transcriptional regulatory protein [YBR239C]	*Saccharomyces cerevisiae*	Score = 127 bits (319)	Expect = 1e-27	111	180
612	Hypothetical protein [CNJ00300]	*Cryptococcus neoformans*	Score = 121 bits (304)	Expect = 7e-26	116	182

The BLAST was conducted using the *N. crassa* AOD5 predicted protein sequence to search the EXPASY/UniProtKB complete databases 24/1/06

Figure 2. The tyrosinase plate assay. Conidia were harvested and spread on non-inducing and chloramphenicol-containing plates. Conidia on non-inducing plates were allowed to grow at 30°C for 2-3 days while conidia on chloramphenicol-containing plates were allowed to grow for 3-4 days. Tyrosine solution was then added and the plates were incubated for 6 h at 30°C. Strain T11-76 is the reporter strain. Colonies on plates containing chloramphenicol, but not those on the non-inducing plate, turn brown after incubation with the tyrosine solution. Mutants *aod-5, aod-6, aod-7* and *aod-8* remain white in the absence or presence of chloramphenicol (see Table 7 for naming of mutants).


Uninduced
Chloramphenicol
T11-76
aod-5
aod-6
T11-76
L2-40
L2-40
L2-62
L2-62

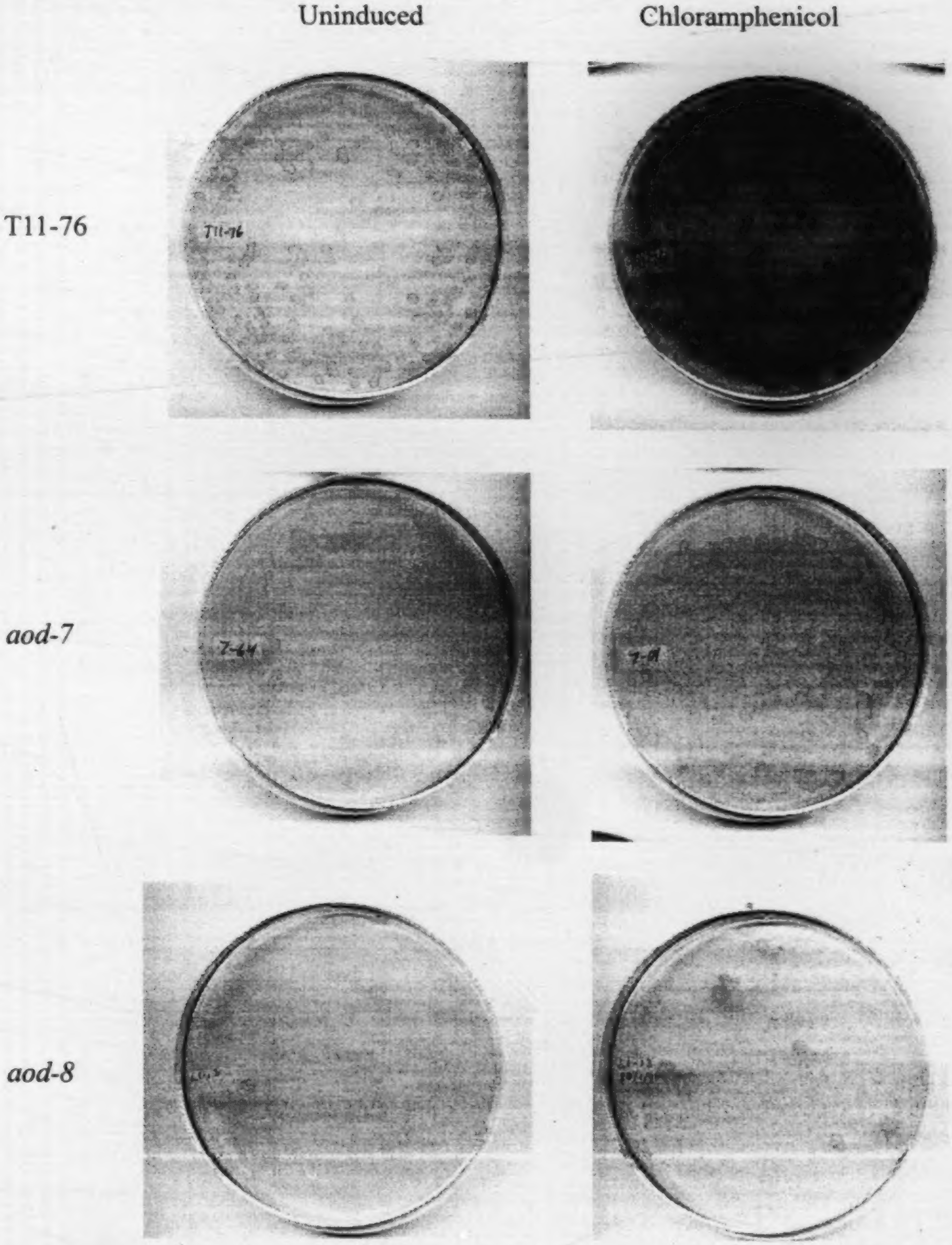

Uninduced
Chloramphenicol
T11-76
T11-76
aod-7
7-64
aod-8

Figure 3. Respirometer Tracings. Alternative oxidase activity was assayed by measuring oxygen consumption over time. Respiration was tested in non-induced cultures and cultures grown in the presence of chloramphenicol or antimycin A to induce alternative oxidase. The presence of alternative oxidase activity was assayed by the addition of potassium cyanide (KCN) at the points indicated by the arrows. An arrest in oxygen consumption indicated that there was no alternative oxidase activity, while a continued decrease in oxygen levels in the presence of KCN indicated alternative oxidase activity. Alternative oxidase is specifically inhibited by the addition of salicylhydroxamic acid (SHAM). Only the reporter strain T11-76 and mutants *aod-6* and *aod-8* are able to grow in the presence of antimycin A.

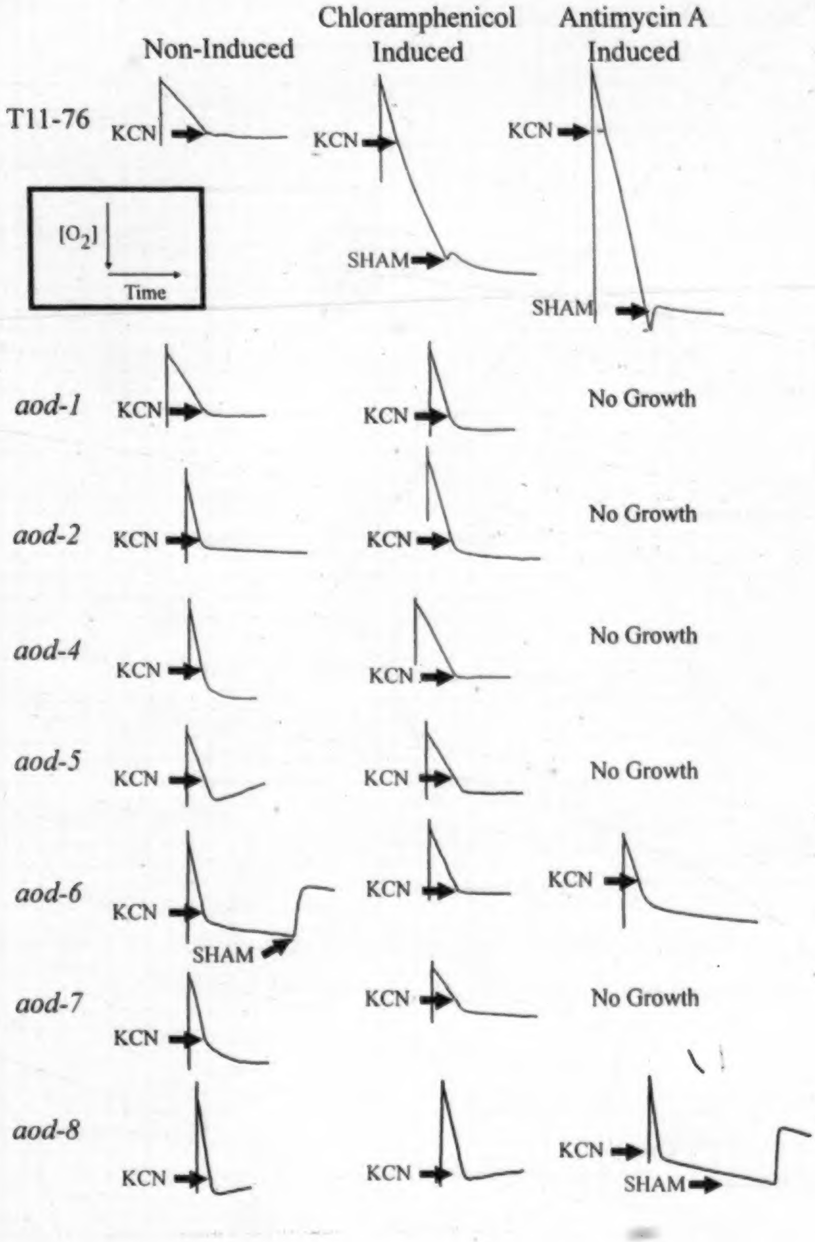

Non-Induced
Chloramphenicol Induced
Antimycin A Induced
T11-76
KCN
KCN
SHAM
KCN
SHAM
[O2]
Time
aod-1
KCN
KCN
No Growth
aod-2
KCN
KCN
No Growth
aod-4
KCN
KCN
No Growth
aod-5
KCN
KCN
No Growth
aod-6
KCN
SHAM
KCN
KCN
aod-7
KCN
KCN
No Growth
aod-8
KCN
KCN
KCN
SHAM

Figure 4. Screening for the integrated reporter. A. PCR primers FNA88 and ADE19 amplify a 1.5 kbp region of the reporter containing the tyrosinase coding sequence and *aod-1* promoter sequence. PCR products were electrophoresed on a 0.8% agarose gel containing ethidium bromide. The arrowhead indicates the λ *HindIII* 2.3 and 2.0 kbp marker bands, while the arrow indicates the reporter PCR product. Control strain NCN233 lacks the reporter and no PCR product was produced. The reporter strain T11-76 gives a single band of 1.5 kbp. NL40-5 and NL40-14 are progeny from the cross L2-40X763. NL40-5 contains the reporter and this PCR produces a band, while NL40-14 lacks the reporter and no band is produced. B. Primers FNA88 and ao5 amplify a 1.3 kbp region of the endogenous *aod-1* gene. PCR products were electrophoresed on a 0.8% agarose gel containing ethidium bromide. Since all strains contain an endogenous copy of *aod-1* this PCR produces a 1.3 kbp band from all four strains. The arrowhead indicates the λ *HindIII* 2.3 and 2.0 kbp marker bands, while the arrow indicates the endogenous *aod-1* PCR product.

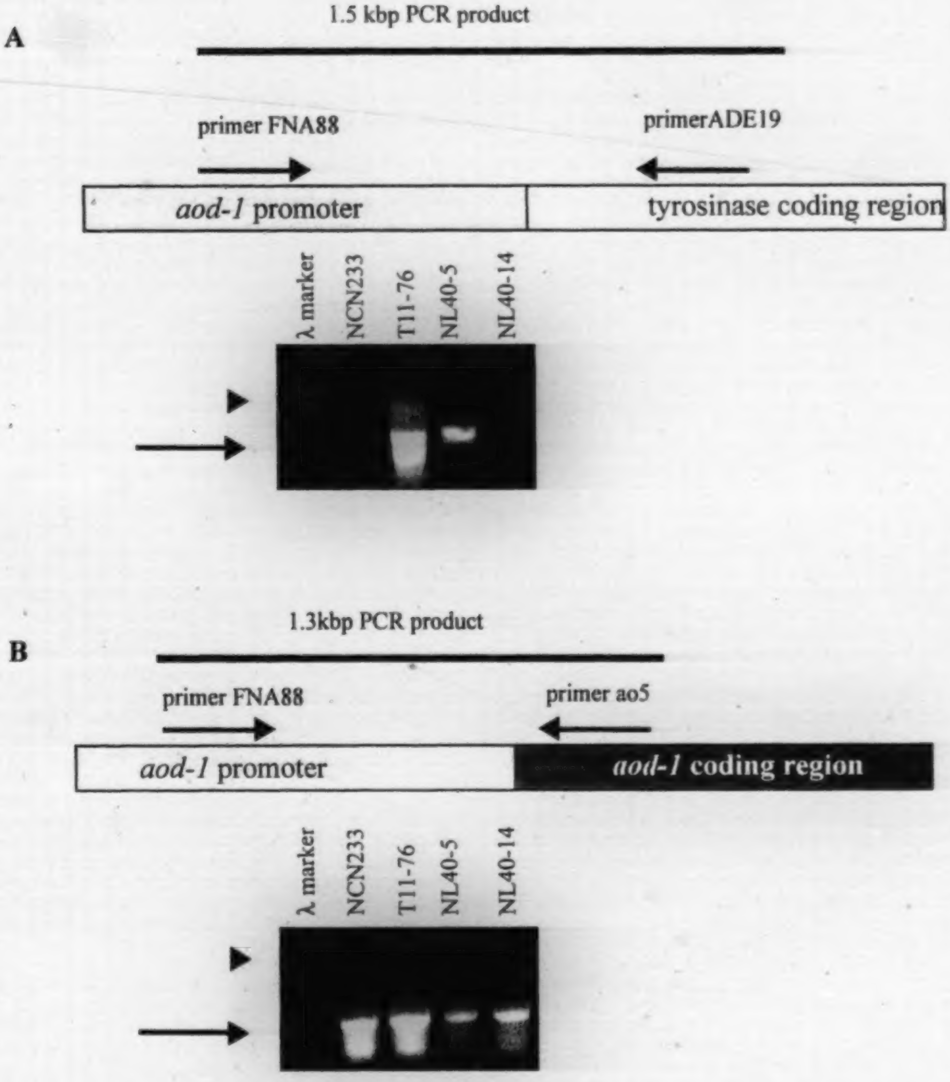
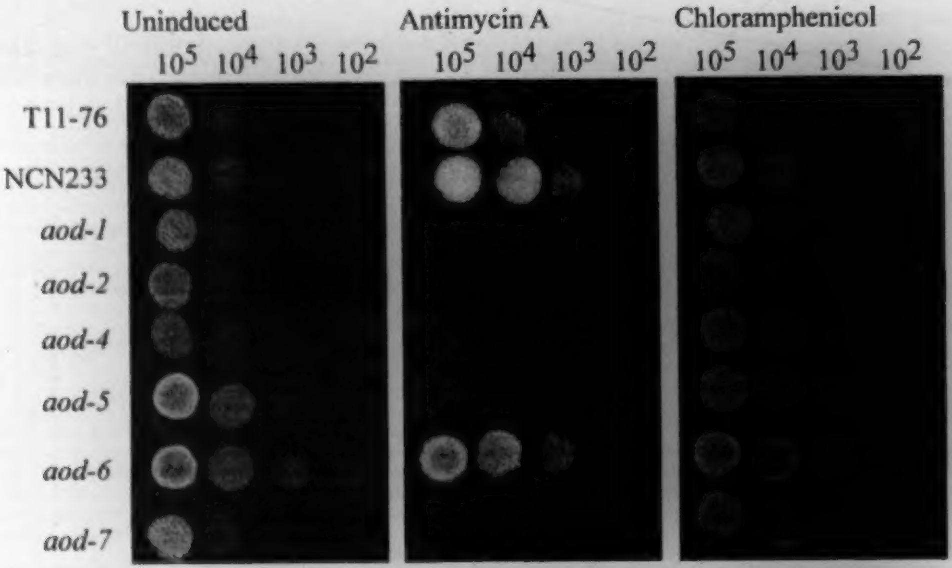
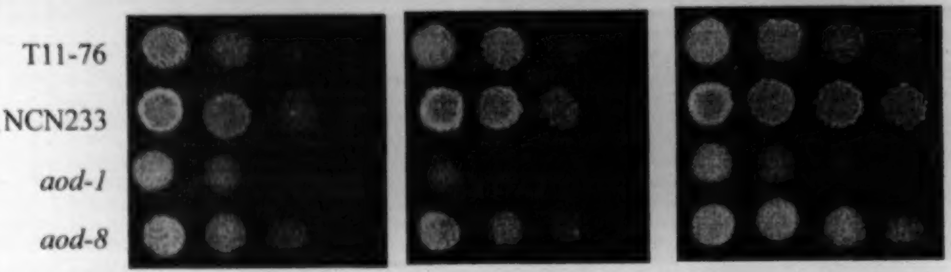

A
1.5 kbp PCR product
primer FNA88
primerADE19
aod-1 promoter
tyrosinase coding region
λ marker
NCN233
T11-76
NL40-5
NL40-14
B
1.3kbp PCR product
primer FNA88
primer ao5
aod-1 promoter
aod-1 coding region
λ marker
NCN233
T11-76
NL40-5
NL40-14

Figure 5. Growth rates of mutant strains. 10 μl of conidia were spotted onto non-inducing medium containing necessary nutritional supplements as well as medium containing antimycin A or chloramphenicol. The number of conidia spotted is indicated above each column. Plates were incubated at 30°C for the indicated times. The difference in relative growth rates of T11-76 and NCN233 between the upper and lower panels is due to the presence of tryptophan in the medium in the upper, but not the lower panel. The *aod-6* strain is auxotrophic for tryptophan, but the presence of this amino acid affects the growth of T11-76. The cause of this effect is unknown, but may be related to the tyrosinase mutation.


Uninduced
Antimycin A
Chloramphenicol
10^5 10^4 10^3 10^2
10^5 10^4 10^3 10^2
10^5 10^4 10^3 10^2
T11-76
NCN233
aod-1
aod-2
aod-4
aod-5
aod-6
aod-7

Uninduced 20.5 hr Antimycin A 93 hr Chloramphenicol 43.5 hr


T11-76
NCN233
aod-1
aod-8

Uninduced 21 hr Antimycin A 47 hr Chloramphenicol 47 hr

Figure 6. Cytochrome spectra. Cytochrome spectra were obtained for the reporter and mutant strains grown under non-inducing conditions or in the presence of chloramphenicol or antimycin A. The spectrum was produced by scanning a reduced *vs.* an oxidized reference from 650 to 500 nm. The peaks corresponding to cytochromes aa_3 (605 nm), *b* (560 nm), and *c* (550 nm) are indicated above the spectrum shown for the T11-76 tracings. Strains *aod-1*, *aod-2*, *aod-4*, *aod-5*, and *aod-7* did not grow in the presence of antimycin A.

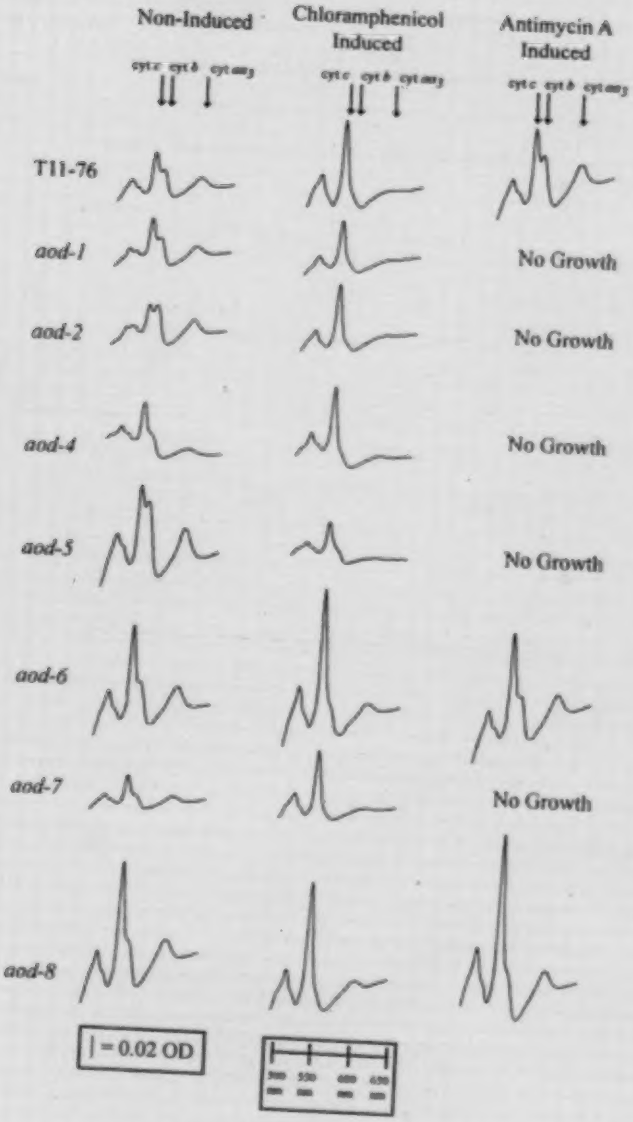

Non-Induced
Chloramphenicol Induced
Antimycin A Induced
cyt c
cyt b
cyt aa3
T11-76
aod-1
aod-2
aod-4
aod-5
aod-6
aod-7
aod-8
No Growth
No Growth
No Growth
No Growth
No Growth
| = 0.02 OD
500
550
600
650

Figure 7. Northern and Western blot analysis of alternative oxidase expression. (A) Ethidium bromide-stained gels of total RNA (5 μg per lane). RNA was isolated from the strains indicated and electrophoresed on a 1% agarose gel. Cultures were uninduced, or induced by growth in the presence of chloramphenicol (CAP) or antimycin A (Ant A). Strains NCN246, T11-76 and NCN233 served as controls as they are wild-type for induction of alternative oxidase activity. NCN246 and NCN233 are also wild-type for *aod-1* regulation while T11-76 is wild-type for *aod-1* regulation except for the accumulation of *aod-1* mRNA under non-inducing conditions. (B) The gels from A were blotted to nylon membranes and probed with a ^{32}P-labeled 1.3 kbp cDNA copy of *aod-1*. The band recognized is 1.3 kbp. (C) Mitochondria were isolated from cultures grown under identical conditions to those from which RNA was isolated. 25 μg of mitochondrial protein from each sample was subjected to SDS-PAGE. The gels were blotted to nitrocellulose and immunodecorated with antiserum to Tom70 (a 70-kDa mitochondrial outer membrane protein), which served as a loading control, or antiserum against the AOD1 protein (36 kDa). A similar figure appears in the thesis of Andrea Todd (Descheneau).

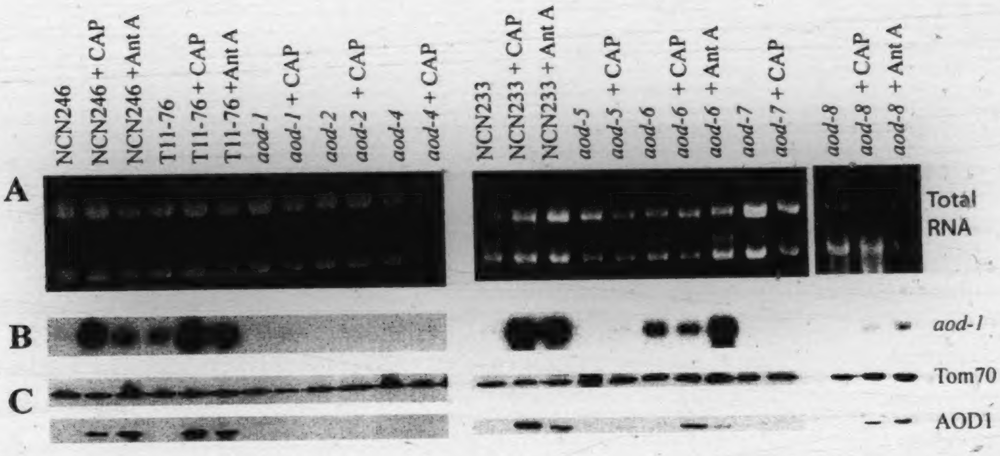

NCN246
NCN246 + CAP
NCN246 +Ant A
T11-76
T11-76 + CAP
T11-76 +Ant A
aod-1
aod-1 + CAP
aod-2
aod-2 + CAP
aod-4
aod-4 + CAP
NCN233
NCN233 + CAP
NCN233 + Ant A
aod-5
aod-5 + CAP
aod-6
aod-6 + CAP
aod-6 + Ant A
aod-7
aod-7 + CAP
aod-8
aod-8 + CAP
aod-8 + Ant A
A
Total RNA
B
aod-1
C
Tom70
AOD1

Figure 8. AOD1 protein in the *aod-6* strain. Cultures of the *aod-6* strain grown in the presence of chloramphenicol lack cyanide-insensitive respiration (A), but do accumulate AOD1 protein (B). The amount of protein relative to a control strain (NCN233) can be somewhat variable as demonstrated in these two different cultures. 25 µg of mitochondrial protein from each sample was subjected to SDS-PAGE. The gels were blotted to nitrocellulose and immunodecorated with antiserum to Tom70 (a 70-kDa mitochondrial outer membrane protein), which served as a loading control, or antiserum against the AOD1 protein (36 kDa).

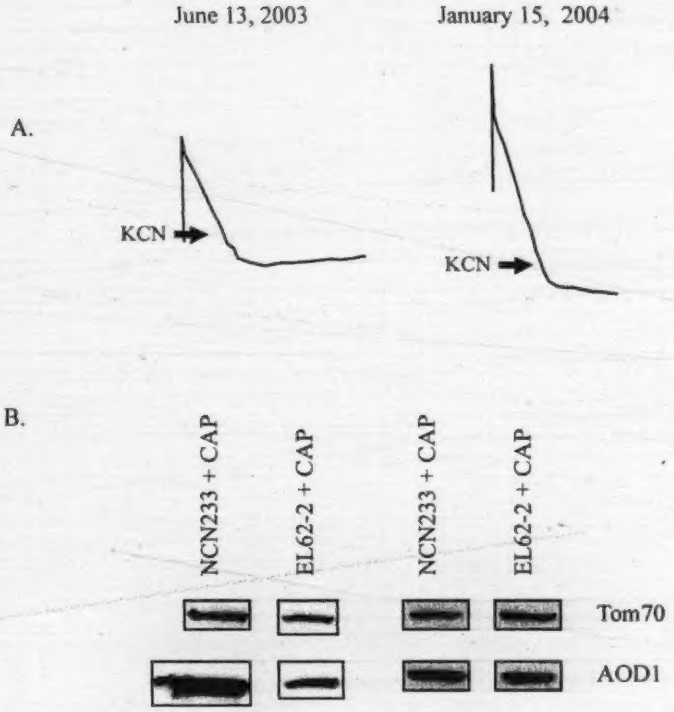

June 13, 2003
January 15, 2004
A.
KCN
KCN
B.
NCN233 + CAP
EL62-2 + CAP
NCN233 + CAP
EL62-2 + CAP
Tom70
AOD1

Figure 9. Nucleotide sequence of *N. crassa aod-5* and predicted amino acid sequence. The predicted coding sequence is shown in uppercase, while upstream and downstream sequence, and the single predicted intron are in lowercase. Numerals in normal font designate nucleotides and italicized numerals indicate amino acids. The *Xba*I sites used to clone the gene are in bold print. The predicted translational start site is underlined. The mutation found in the *aod-5* mutant strain in codon 512 is indicated above the sequence and is boxed.

```
cgcctctctagagccggctcagagcccattggccgttctgctggagcggacagcaagtcc  -660
agtgagaatacgagacgacgtgctggtcattgtgtatcaggcctcgtcagctggagggca  -600
gtcatttgtcggcgagtccatcgtcaacacgtcccagactatattgcggaagcccacagt  -540
cccggcacagtatcaaagagacaagagccttggatgccatatctggtctcgcacgcattg  -480
tgttccgttatcccccgcctcgccgccgcccaccactgccgctcatcaccatcttcacta  -420
ctaccctgcttaattgaggcgccgttgattttgccacacgtacatcgcccaccgccaaat  -360
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ctcttcagaaaaggtgccaccaccgtgggattgctgcaggagagctttgggtctcttgaa  -240
gcttgttccctctctctgacccccatatcggacctgatacgtctctgctagcctggtccc  -180
tacctgcttcagagctcccgcgtccttgcctgccagattgcctgcctctctgcctgccct  -120
gtctcgtctcgcaatctagttggctgctttgggcgtctcctggcacattctgcactaccc  -60
tgccaccactctctgtcttgactccaagaccctgtctgtatatatagagctaaggcacg   -1

ATG CCG GAC GAC GTT GGA CCC GCC GAG GCA GAG GTC TCG GGT GCT    45
 M   P   D   D   V   G   P   A   E   A   E   V   S   G   A     15

GTG TCT GAG AGC GAC AAC GAG TAC GAC GAA ACC GAG GTC ACT ACC    90
 V   S   E   S   D   N   E   Y   D   E   T   E   V   T   T     30

AAG GAC GAC GAC GAC GAA AAG ATG GCC GAA CGA TCC GTT GCT TCC   135
 K   D   D   D   D   E   K   M   A   E   R   S   V   A   S     45

GAA GGT GTC GAA ACC AAC GGT GAC CAG AAG AAG AAG TAT GAC CCC   180
 E   G   V   E   T   N   G   D   Q   K   K   K   Y   D   P     60

AAG GAT CCC TTG CGG CCC AGG AGG AAA AAA GCC CGG AGA GCC TGC   225
 K   D   P   L   R   P   R   R   K   K   A   R   R   A   C     75

TAT GCG TGT CAG AGA GCC CAT TTG ACC TGC Ggtaagttttacttttgct   275
 Y   A   C   Q   R   A   H   L   T   C                         85

cacttcagtccactgtttccccaagttagctttccttgccataaacggatacggcacgga  335
aggaaactaccatcacctcgattctccgtcccctttcgcatgtcacgaaaagaaaaaaa  395
aaaaaaaaaaaaaaaaaaaaaaaccccacgatgatgctaatgttgactttggtttagGT  454
                                                          G    86

GAC GAA CGA CCA TGC CAA CGG TGC ATC AAA CGT GGA TTA GCT GAA   499
 D   E   R   P   C   Q   R   C   I   K   R   G   L   A   E    101

GCT TGC CAG GAC GGT GTC CGG AAG AAG GCA AAG TAT CTC CAC GAT   544
 A   C   Q   D   G   V   R   K   K   A   K   Y   L   H   D    116

GCT CCC CCG GAA GCC TTA CGC CCC GTA CTT GGA CCC AAC TAC AAT   589
 A   P   P   E   A   L   R   P   V   L   G   P   N   Y   N    131

CCT GCT GCC GCT GTA TCT GTC CGG AAT GGC CAT CGG CAT CCT TCT   634
 P   A   A   A   V   S   V   R   N   G   H   R   H   P   S    146

AAC GCT GGG TCG GAT GCT GGA TCT TCT ATT GGT ACC TTC TAC TCG   679
 N   A   G   S   D   A   G   S   S   I   G   T   F   Y   S    161

CAG TCG ACT CAG TAT CCA GTC TTC TCG TCT GCA GCA ACA CAA TTA   724
 Q   S   T   Q   Y   P   V   F   S   S   A   A   T   Q   L    176
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GGA TCA ATA CCG GAG AAC TTG CCC TTC CCC CAG CAA TCT CCC GTA   769
 G   S   I   P   E   N   L   P   F   P   Q   Q   S   P   V    191

TCG CCA ACT TTC CAG CCC TCT AGC AAC CCT CAG CTC GGT TCT ATT   814
 S   P   T   F   Q   P   S   S   N   P   Q   L   G   S   I    206

GGG GTC AGT TCA GTT TCC AGC CCT ATG AAT AGC TTC CCT CCA GCG   859
 G   V   S   S   V   S   S   P   M   N   S   F   P   P   A    221

CTA TTT GAC CCG AGT AAT CCT GCC ATT TTC AAC TTC AAT TTG GAG   904
 L   F   D   P   S   N   P   A   I   F   N   F   N   L   E    236

GGA CTC AAC TTT GGA AGC CAG TAC GGC GCC ATG GAG TTT GGT ATG   949
 G   L   N   F   G   S   Q   Y   G   A   M   E   F   G   M    251

CTG GGC CAC ATG TCC TCG GGC GCT GCT GAG ACT CCT CCG CGG GAT   994
 L   G   H   M   S   S   G   A   A   E   T   P   P   R   D    266

CCG TCC ATG GCA CAG CAG GGC ACT AGT GAT GTC GGA TTC AAC CCC  1039
 P   S   M   A   Q   Q   G   T   S   D   V   G   F   N   P    281

TCT GGA GTC TTT GGA AAC GGT CTG AAC CAG TTC GAA AAG GTT TAT  1084
 S   G   V   F   G   N   G   L   N   Q   F   E   K   V   Y    296

GAC AAT AAC ACC GGT TTG ATA AGC GAC TTC CTG ACT CTG GAT GCC  1129
 D   N   N   T   G   L   I   S   D   F   L   T   L   D   A    311

CAT TCC AAT GGC TTG TAC TCC CAA GGA AAC CTC CAA CAT GGT CTA  1174
 H   S   N   G   L   Y   S   Q   G   N   L   Q   H   G   L    326

CCG CAT GCC TAT GCT ATC CCC GCT GGT CCC ACT AGT CTG CAA AGC  1219
 P   H   A   Y   A   I   P   A   G   P   T   S   L   Q   S    341

CCT AGT ACC GAG AAC AAC AGT CCG CAA CCG ACA GGA TTT GGT TTT  1264
 P   S   T   E   N   N   S   P   Q   P   T   G   F   G   F    356

GAG TCT CCC ACC GCC ACC AAC TAC ACG GGG GTT CCA GGT GCT GCA  1309
 E   S   P   T   A   T   N   Y   T   G   V   P   G   A   A    371

GGA AAC CAG CCG GGC TCC CAG CAG CCA CGG GCG CAG AAA CCA AAG  1354
 G   N   Q   P   G   S   Q   Q   P   R   A   Q   K   P   K    386

ACG CCG GCC TTG GGG AAG CTC GGG CCA CAG TCA GTT CTG GGT AAG  1399
 T   P   A   L   G   K   L   G   P   Q   S   V   L   G   K    401

AGG CAG CGA GAT CCA TCA TCT ATC TAC GAG GCA GTC AAG GAA CCA  1444
 R   Q   R   D   P   S   S   I   Y   E   A   V   K   E   P    416

TTC CAA TAT GTG GCT AGT TTC CAC AAG CTC ATC AGT CTA CTT CAG  1489
 F   Q   Y   V   A   S   F   H   K   L   I   S   L   L   Q    431

AAC CGA TTT TCG GGC GCG AGC ACG ATC AGC ATC GTA AGG TCA CTA  1534
 N   R   F   S   G   A   S   T   I   S   I   V   R   S   L    446
```

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GCC AGC ATT CGC CCA TCT TTC ATG TCA TGT ATG AAA ACG CTC AAC  1579
 A   S   I   R   P   S   F   M   S   C   M   K   T   L   N    461

AGG GCC GAC CTC ATC TTC ATG GAG AAA AGC TTC CAG CGT GCC CTT  1624
 R   A   D   L   I   F   M   E   K   S   F   Q   R   A   L    476

TTC GAG CAT GAG GAG TTC ATG CAC CAA TCG CCA AGC CCG GCC ATC  1669
 F   E   H   E   E   F   M   H   Q   S   P   S   P   A   I    491

GCT TGC CGT CGA ACC GGC GAA ATA GCC GCC GTT AAC AAG GAG TTC  1714
 A   C   R   R   T   G   E   I   A   A   V   N   K   E   F    506
                      A
ACG GCG CTA ACA GGG TGG ACA AAG GAT GTG CTT CTT GGG AAG ACG  1759
 T   A   L   T   G   W   T   K   D   V   L   L   G   K   T    521

CTA AAT CTC AAT GCG AAT ATG GGT GGC ACT AAC TCG GAT ACT CTT  1804
 L   N   L   N   A   N   M   G   G   T   N   S   D   T   L    536

TCC ATC AGC AGC AAA GGA GGC AGA GGA GGC ATT GTG GGA ACA ACA  1849
 S   I   S   S   K   G   G   R   G   G   I   V   G   T   T    551

CCC AGG CTA AAA CCT TTG CAT CCA GAA CAG GGC ACG AAC GCC GAC  1894
 P   R   L   K   P   L   H   P   E   Q   G   T   N   A   D    566

AGT CAG CAA CAG CAA TCG CAG CAA CAC AAG GAA CAA CCA CAA CCG  1939
 S   Q   Q   Q   Q   S   Q   Q   H   K   E   Q   P   Q   P    581

GTA TTC CTT GCG GAG CTC ATG GAC GAA GCG AGC GTG ACG CAG TTC  1984
 V   F   L   A   E   L   M   D   E   A   S   V   T   Q   F    596

TAC GAA GAT TAC GCC CAG CTA GCG TTT ACA CAC AGC CGA GGT ACA  2029
 Y   E   D   Y   A   Q   L   A   F   T   H   S   R   G   T    611

GTG GTG AGA AAA TGC CGA CTT CTC AAG TAC CGC ACC CAA GAG AAC  2074
 V   V   R   K   C   R   L   L   K   Y   R   T   Q   E   N    626

ATG GAC GCC GCC GCC GCC GCG GCT GCT GCT GCT TCT GCT CCC ACA  2119
 M   D   A   A   A   A   A   A   A   A   A   S   A   P   T    641

GCT TCA GGG GGT AGT GGT AGC AGT AAC GGC ACC GTT GTC AAT GGA  2164
 A   S   G   G   S   G   S   S   N   G   T   V   V   N   G    656

GGG CCA GAT AGC AGC CCA GCC GGA AAG ACA GAG AAA GAA CGA CCG  2209
 G   P   D   S   S   P   A   G   K   T   E   K   E   R   P    671

ACG GGC GTG AAT GTT GCC AGC AAT AGT ATC TTG AGC AAC AGG GTC  2254
 T   G   V   N   V   A   S   N   S   I   L   S   N   R   V    686

GCC AAG ATC GAC GGA GAA CAC GGT ATC TCG AAA CTG GAA AGA GAT  2299
 A   K   I   D   G   E   H   G   I   S   K   L   E   R   D    701

GGT AAG CTA GAA TGC AGC TAT ACG TGG ACC ATC AAG CGG GAT GTG  2344
 G   K   L   E   C   S   Y   T   W   T   I   K   R   D   V    716
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TTT GAT ATA CCC ATG ATT ATC ATG ATC AAC GTA AGT GGT GTC TGA 2389
F D I P M I I M I N V S G V * *730*

cgggcctttttctcttttcgcccttttcttctttgttttttccatcccaattcgctttccc 2449
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accagctagctgtatgattagaaccagtactgctg**tctaga**acaaacaagacattcattg 2749

Figure 10. Northern analysis of *aod-1* expression over time. Upper Panel: Ethidium bromide-stained gels of total RNA. RNA was isolated from the cultures indicated and electrophoresed on a 1% agarose gel (5 μg per lane). Six cultures of a wild-type strain (NCN233) were grown for approximately 16 h under non-inducing conditions. Antimycin A was then added to five of the cultures, which were allowed to continue to grow in the presence of antimycin A for the times indicated. Lower Panel: The gel from A was blotted to a nylon membrane and probed with a ^{32}P-labeled 1.3 kbp cDNA copy of *aod-1*.

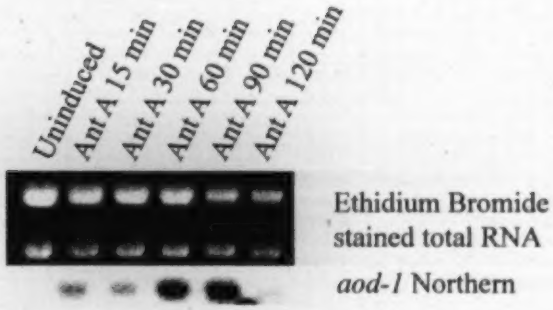

Uninduced
Ant A 15 min
Ant A 30 min
Ant A 60 min
Ant A 90 min
Ant A 120 min
Ethidium Bromide stained total RNA
aod-1 Northern

Figure 11. RT-PCR. Ten cultures of a control strain (NCN233), which is wild-type for alternative oxidase induction, were grown for approximately 16 h under non-inducing conditions in 250 ml liquid Vogel's medium. Antimycin A was then added to a final concentration of 0.5 μg/ml to nine of the cultures, which were allowed to continue to grow in the presence of antimycin A for the times indicated. Cultures indicated by an asterisk were grown for 24 h and contained the indicated inhibitor from the start of growth. The *aod-5* uninduced culture was grown for 16 h and the *aod-5* culture with chloramphenicol was grown for 24 h. (A) Mitochondria were isolated from the same cultures from which the RNA was derived. 25 μg of mitochondrial protein from each sample was subjected to SDS-PAGE and the gel was blotted to nitrocellulose. Proteins were immunodecorated with antiserum to Tom70 (a 70-kDa mitochondrial outer membrane protein), which serves as a loading control, or antiserum against the AOD1 protein (36 kDa). (B-E) Total RNA was isolated from the same cultures from which the mitochondria were derived. cDNA was reverse transcribed using an oligo-dT20 primer. Gene-specific primers were then used for PCR. Controls for the RT-PCR are in the last three lanes on the right of the gel and were treated as the rest of the samples, but with the indicated changes. The arrow on the left indicates the position of the 456 bp lambda *Hind*III marker fragment. (B) Ethidum bromide-stained PCR products from primers specific to *Tim-8* (FNA 272 and FNA 273), a gene encoding a component of the mitochondrial import machinery which is shown here as a control. As predicted, these primers produced a 529 bp band from cDNA and a 791 bp band from genomic DNA (gDNA). One tenth of the total volume of the PCR reaction was electrophoresed on a 1.6% gel. (C-E) Ethidum bromide-stained PCR products from primers specific to *aod-5* (ICL1 and ICL9). These primers were predicted to produce a 405 bp band from a cDNA template and a 601 bp band from a gDNA template (see panels F gDNA, and G, cDNA for schematic). One half of the total volume of the PCR reaction was electrophoresed on a 1.6% agarose gel. Panels C, D, and E represent three separate trials of PCR reactions conducted using the same cDNA template. The upper band (indicated by the black arrowhead on the right) in all three panels is a PCR product from gDNA. This was confirmed by sequence analysis, which indicated the presence of intronic sequence. The lower band (indicated by the white arrowhead on the right) in all three panels is a PCR product from cDNA. This was also confirmed by sequence analysis, which indicated the absence of intronic sequence.

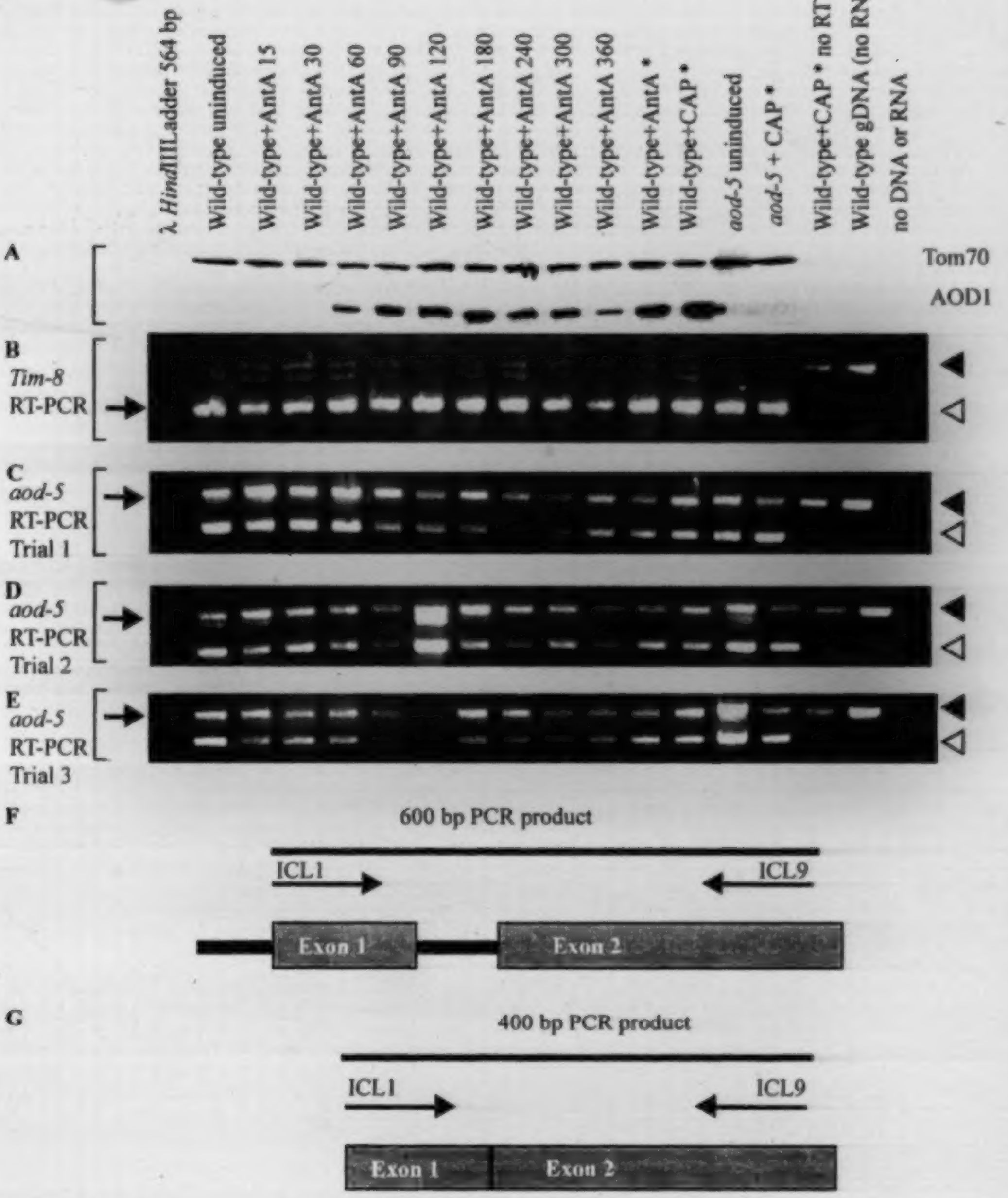
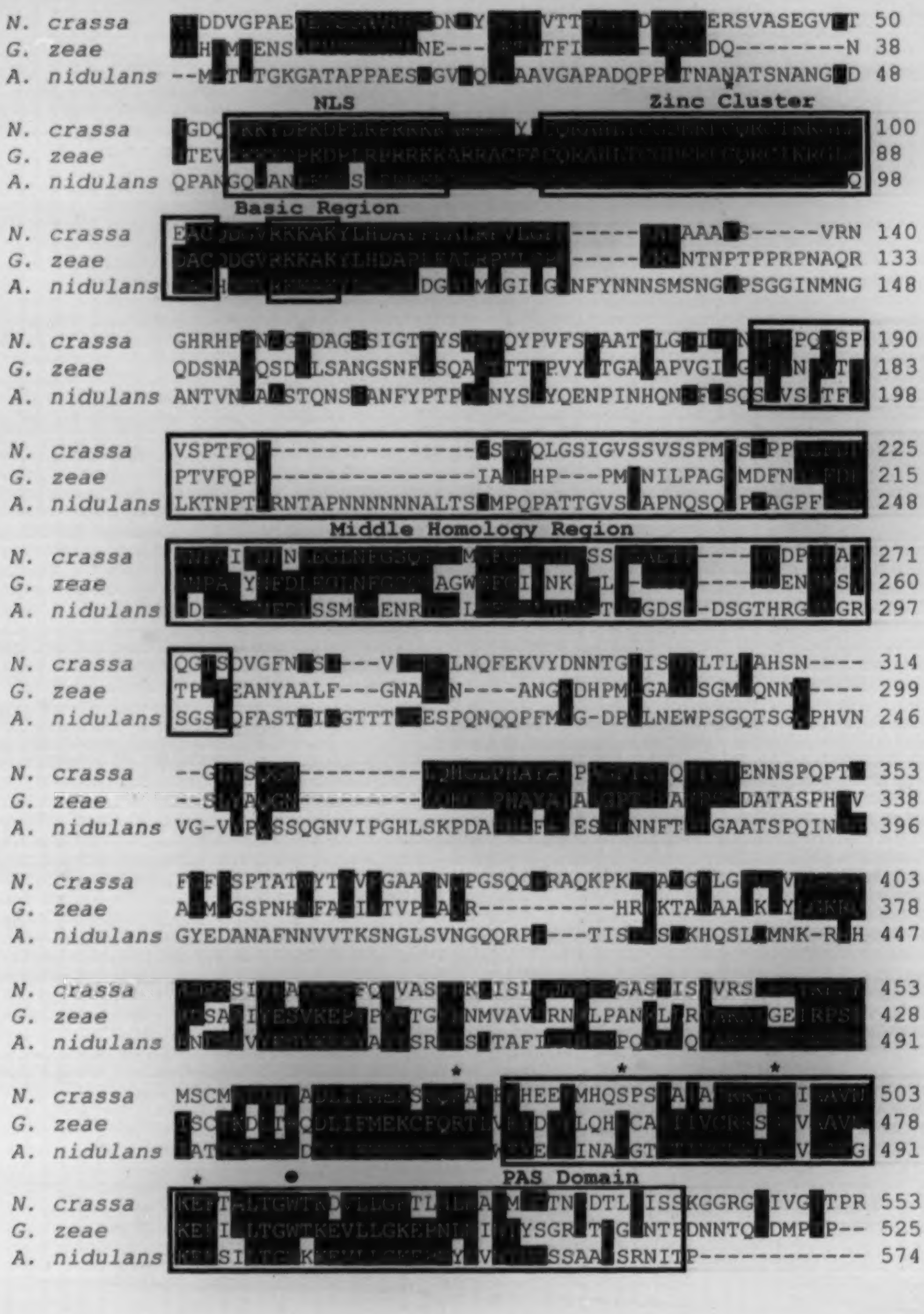

λ HindIIILadder 564 bp
Wild-type uninduced
Wild-type+AntA 15
Wild-type+AntA 30
Wild-type+AntA 60
Wild-type+AntA 90
Wild-type+AntA 120
Wild-type+AntA 180
Wild-type+AntA 240
Wild-type+AntA 300
Wild-type+AntA 360
Wild-type+AntA *
Wild-type+CAP *
aod-5 uninduced
aod-5 + CAP *
Wild-type+CAP * no RT
Wild-type gDNA (no RNA)
no DNA or RNA
A
Tom70
AOD1
B
Tim-8
RT-PCR
C
aod-5
RT-PCR
Trial 1
D
aod-5
RT-PCR
Trial 2
E
aod-5
RT-PCR
Trial 3
F
600 bp PCR product
ICL1
ICL9
Exon 1
Exon 2
G
400 bp PCR product
ICL1
ICL9
Exon 1
Exon 2

Figure 12. Alignment of homologous AOD5 sequences. The predicted protein sequences of AOD5 proteins from *N. crassa, G. zeae* and *A. nidulans* are aligned. Amino acids conserved between at least two species are shaded. Conserved domains are boxed and labeled, included the Nuclear Localization Signal (NLS), Zinc Cluster DNA binding domain, basic region, Middle Homology Region (MHR) and PAS domain. Amino acid numbers are indicated to the right. Residues changed by site-directed mutagenesis are indicated by asterisks. The residue changes are C91S, ΔR474, ΔS488^{FS}, ΔG497, G497A and ΔE505. The site of the premature stop codon in the *aod-5* mutant strain is indicated by the black circle.


N. crassa
G. zeae
A. nidulans
NLS
Zinc Cluster
Basic Region
Middle Homology Region
PAS Domain

```
N. crassa     LKPLHPEQGTNADS[illegible]QQQ[illegible]QQHKEQ[illegible]M[illegible]EA[illegible]TQ[illegible]Y[illegible]Q[illegible] 603
G. zeae       -------------RP[illegible]KATLDN[illegible]N[illegible]QFVF[illegible]G[illegible]Q[illegible] 564
A. nidulans   ----------------RS[illegible]VE[illegible]TG[illegible]H[illegible] 613

N. crassa     A[illegible]TH[illegible]T[illegible]V[illegible]K[illegible]Q[illegible]N[illegible]AAAAAAASAPTASGGSGSSNGTV 653
G. zeae       [illegible]E[illegible]SR[illegible]K[illegible]Q[illegible]S[illegible]R[illegible]NKY[illegible]SSQSL[illegible]IKSEHGVPKDIQPG----------- 503
A. nidulans   A[illegible]G[illegible]S[illegible]TTR[illegible]K[illegible]K[illegible]K[illegible]D[illegible]E[illegible]QSDDNGQRWNN------------ 651

N. crassa     VNGGPDSSPAGKTEKERPTGVNVASNS[illegible]N[illegible]AK[illegible]G[illegible]K[illegible]-[illegible] 702
G.zeae        ---------------------------[illegible]S[illegible]TR[illegible]S[illegible]RI[illegible]-[illegible] 625
A. nidulans   ------------------------------HL[illegible]KGG[illegible]AN[illegible]A[illegible]MNQ[illegible]GFK[illegible] 672

N. crassa     KLE[illegible]SYT[illegible]IKRDVFDIP[illegible]I[illegible]MIN[illegible]SGV---------------------- 730
G. zeae       KVECTYCWTIKRDVFDIP[illegible]M[illegible]I[illegible]FLPRYLPDQGPQQLAV---------- 665
A. nidulans   KV[illegible]CA[illegible]V[illegible]L[illegible]V[illegible]RLPLP-------------------- 702
```

Figure 13. Nucleotide sequence of *A. nidulans aod-5* and predicted amino acid sequence. The predicted coding sequence is shown in uppercase, while upstream and downstream sequence, and the single predicted intron are in lowercase. Numerals in normal font designate nucleotides and italicized numerals indicate amino acids. The locations of the two oligonucleotides used to amplify this sequence are in bold print, although the lower strand sequence was used for the 3' oligo. The predicted translational start site is underlined.

gcccttcgaaagaccgttaaatcgctcacct**ccaaacggtctctgtctccc**cgcagcacc -521
gcccggcaagtgtgagtgtgagtctcacgcttccatccgcccgccgccaagtactcccac -480
actttttttttatgattaattcttgctgatgtgctaatgaaacaaggcacgttgctgtcc -420
ctaggtagttttagtttggctgagcgcgatttgtctccaatttcacaatcacattcctt -361
ctccacggactgttcccaaactttctttttctcctagtcgtgtcttcccctcccttctta -301
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gcggtgacgacgacgaagccgattctcagagcgtgacgacccatttccggggagaagcct -121
ttaaccgatcccgtaccgatctgtcaccccggattcgtcacttcaatagtccagacgaac - 61
gacgcatcccgccacctcttcggtccttttcatcatcgcgagcttgtcgattaatggaaa - 1

ATG GAG ACG GAG ACA GGC AAG GGC GCT ACT GCT CCC CCT GCA GAA 45
M E T E T G K G A T A P P A E *15*

TCT AGT GGG GTC GAA CAA GAT ACC GCA GCC GTT GGC GCG CCC GCC 90
S S G V E Q D T A A V G A P A *30*

GAC CAG CCG CCC AAG ACT AAT GCG AAC GCG ACT TCG AAT GCG AAC 35
D Q P P K T N A N A T S N A N *45*

GGC GAA GAT CAG CCG GCC AAC GGC CAG AAA GCG AAC CCG AAG GAT 80
G E D Q P A N G Q K A N P K D *60*

CCG TCA AGA CCG AGG AGG AAG AAG GCG CGG CGC GCC TGT TTT GCT 125
P S R P R R K K A R R A C F A *75*

TGT CAG CGG GCG CAT CTA ACG TGC Ggtacgctcctgccctgctcaggtaccc 152
C Q R A H L T C *83*

gtgtcctgggcgcgccaactaacagagtgtgtagGT GAT GAG AGA CCA TGT 203
G D E R P C *89*

CAA CGC TGC ATC AAG CGC GGG CTC CAA GAT GCC TGC CAT GAT GGG 248
Q R C I K R G L Q D A C H D G *104*

GTT CGC AAG AAG GCC AAG TAC CTG CAT GAC GCG CCA GAC GGC GCT 293
V R K K A K Y L H D A P D G A *119*

CTG ATG CCT GGG ATC GGC GGC AAT AAT TTC TAC AAC AAC AAT TCA 338
L M P G I G G N N F Y N N N S *134*

ATG TCG AAC GGT GTC CCA TCG GGT GGC ATA AAT ATG AAT GGC GCG 383
M S N G V P S G G I N M N G A *149*

AAT ACC GTC AAT TCA GCG GCG AGT ACG CAG AAT TCG AGC GCA AAC 428
N T V N S A A S T Q N S S A N *164*

TTC TAT CCT ACT CCG CAG TCG AAC TAC AGC TTA TAC CAG GAG AAT 473
F Y P T P Q S N Y S L Y Q E N *179*

CCG ATC AAC CAC CAA AAC TCT TTC CCA TCG CAA TCG CCG GTG TCC 518
P I N H Q N S F P S Q S P V S *194*

CCG ACC TTT AGC TTG AAG ACC AAC CCA ACC CCT CGC AAT ACC GCA 563
P T F S L K T N P T P R N T A *209*

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CCT AAC AAC AAC AAT AAT AAT GCC TTA ACG TCT TCG ATG CCC CAA    608
 P   N   N   N   N   N   N   A   L   T   S   S   M   P   Q     224

CCC GCG ACC ACT GGT GTG TCG AAT GCA CCC AAC CAG AGC CAG AAT    653
 P   A   T   T   G   V   S   N   A   P   N   Q   S   Q   N     239

CCG TTC GCC GGC CCG TTC TTC GAC CCC AGT GAT CCG GCT CTT TTC    698
 P   F   A   G   P   F   F   D   P   S   D   P   A   L   F     254

AAC TTT GAT CTG TCG AGT ATG AAT TTT GAA AAC CGA TAT GGT GCG    743
 N   F   D   L   S   S   M   N   F   E   N   R   Y   G   A     269

CTG GAG TTC GGT ATG CTT GGA CAC ATG GCT ACT GGG GCT GGC GAC    788
 L   E   F   G   M   L   G   H   M   A   T   G   A   G   D     284

TCT CCA GAC TCT GGC ACC CAC AGA GGA TCG ATG GGC CGC AGC GGT    833
 S   P   D   S   G   T   H   R   G   S   M   G   R   S   G     299

TCG ACG CAG TTT GCG TCT ACG CCT ATT GGA GGA ACT ACG ACG TTC    878
 S   T   Q   F   A   S   T   P   I   G   G   T   T   T   F     314

GGC GAG AGC CCG CAG AAT CAG CAG CCA TTC ATG TTC GGG GAT CCG    923
 G   E   S   P   Q   N   Q   Q   P   F   M   F   G   D   P     329

CTA TTG AAC GAG TGG CCG AGT GGA CAG ACC TCG GGC CAA CCG CAC    968
 L   L   N   E   W   P   S   G   Q   T   S   G   Q   P   H     344

GTG AAT GTC GGC GTA TAT CCG CAG TCT AGC CAG GGC AAC GTG ATA   1013
 V   N   V   G   V   Y   P   Q   S   S   Q   G   N   V   I     359

CCG GGA CAT CTG TCT AAG CCT GAT GCG CCT CAC GCT TTC GCA ATT   1058
 P   G   H   L   S   K   P   D   A   P   H   A   F   A   I     374

GAA AGT GGA CCG AAC AAC TTC ACT AGC CCG GGC GCG GCG ACG AGC   1103
 E   S   G   P   N   N   F   T   S   P   G   A   A   T   S     389

CCG CAG ATC AAC AGC GGC GGA TAT GAA GAT GCG AAC GCG TTT AAC   1148
 P   Q   I   N   S   G   G   Y   E   D   A   N   A   F   N     404

AAT GTT GTG ACG AAG TCT AAT GGA CTA TCT GTG AAC GGA CAG CAA   1193
 N   V   V   T   K   S   N   G   L   S   V   N   G   Q   Q     419

CGT CCT CCG ACC ATT TCT ACA CCG AGT TTG AAG CAC CAA AGC CTG   1238
 R   P   P   T   I   S   T   P   S   L   K   H   Q   S   L     434

CAG ATG AAC AAA CGA CGT CAC CGC AAT CCA TCG GCA GTC TAC GAA   1283
 Q   M   N   K   R   R   H   R   N   P   S   A   V   Y   E     449

AGC GTC AAG GAG CCG TAC GCG TAT ACC AGT CGC TTC CAT AGC CTA   1328
 S   V   K   E   P   Y   A   Y   T   S   R   F   H   S   L     464

ACG GCG TTT ATT CAA CGG CGC TTC TCG CCG CAG AAG ACA CTG CAG   1373
 T   A   F   I   Q   R   R   F   S   P   Q   K   T   L   Q     479
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ATT GCC AAA GCC CTG GCA TCT ATT CGA CCT TCA TTT ATT GCA ACA  1418
 I   A   K   A   L   A   S   I   R   P   S   F   I   A   T    494

ACC AAA ACA CTA AAC CGG GAT GAT CTT ATC TTT ATG GAG AAG TGC  1463
 T   K   T   L   N   R   D   D   L   I   F   M   E   K   C    509

TTT CAG CGG ACA CTC TGG GAG TAT GAG GAT TTC ATC AAC GCT TGC  1508
 F   Q   R   T   L   W   E   Y   E   D   F   I   N   A   C    524

GGC ACA CCG ACC ATT GTT TGC CGG CGC ACT GGC GAG GTT GCA GCT  1553
 G   T   P   T   I   V   C   R   R   T   G   E   V   A   A    539

GTT GGG AAA GAA TTT AGC ATC TTG ACG GGA TGG AAG AAA GAG GTA  1598
 V   G   K   E   F   S   I   L   T   G   W   K   K   E   V    554

TTA CTA GGA AAG GAG CCG AAT TAT AAT GTC AAC ACA GGT GGT TCA  1643
 L   L   G   K   E   P   N   Y   N   V   N   T   G   G   S    569

TCG GCC GCA AAT TCC CGA AAC ATC ACG CCA CGT AGC TCC GTT GAA  1688
 S   A   A   N   S   R   N   I   T   P   R   S   S   V   E    584

AGC ACT GGC CGT CCT CAT CCG GTG TTT CTG GCG GAA CTG TTG GAC  1733
 S   T   G   R   P   H   P   V   F   L   A   E   L   L   D    599

GAT GAC AGC GTG GTA GAA TTT TAC GAA GAC TTT GCG CGA TTA GCA  1778
 D   D   S   V   V   E   F   Y   E   D   F   A   R   L   A    614

TTC GGG GAT TCG CGC GGC AGC GTG ACA ACG CGC TGC AAA TTG CTC  1823
 F   G   D   S   R   G   S   V   T   T   R   C   K   L   L    629

AAA TAC AAA ACA AAA GAG GAC ATG GAA GCG GCA CAG TCC GAT GAC  1868
 K   Y   K   T   K   E   D   M   E   A   A   Q   S   D   D    644

AAC GGG CAG CGG TGG AAC AAC CAC CTG CGT AAG GGG GGA ATT GCG  1913
 N   G   Q   R   W   N   N   H   L   R   K   G   G   I   A    659

AAC GAG GCC GGG ATG AAC CAG TTA GGA TTC AAA GAT GGC AAA GTC  1958
 N   E   A   G   M   N   Q   L   G   F   K   D   G   K   V    674

GAG TGC GCC TAC TGC TGG ACA GTC AAG CGC GAC GTG TTC GAC ATC  2003
 E   C   A   Y   C   W   T   V   K   R   D   V   F   D   I    689

CCT ATG TTA ATA GTG ATG AAC GTG CGT CTT CCT CTT CCC TGA accc 2049
 P   M   L   I   V   M   N   V   R   L   P   L   P   *        702
```

gaacttcattatgagctaacattgatcagttcctgccttgtatttgaaaggagagtgtta 2109
ttattgcgccgcttaattcgctgtatatgatttatggtctagttacgggaggaggatacc 2169
cgaaggtttaaaaaaggatttttttcttgtttatacttggatgcatgatggatatgatta 2229
tcttggagctgggatagagcttcttgtgctggtgcattcgctttgagctatctaaggcga 2289
agcccggttttttcaagctaggtgaattcacttcccttctagtcgtagtcgactgcggct 2349
gcgtaggtggtggctcgtgtctcgctcttctctatctcattgtcaacaagcgactgtgta 2409
gcaatttcagcaagctgtaattatccagacttggtcgcccttgtatgcccaacaacaaga 2469
atcctcccccggttgtccccggttgttcaccttaattgcttacggcgagaacccttcaaa 2529
cgtctc**ccaaccgaggccaagaggtg**tttggcgacatataataatgatttccagttaga 2589

Figure 14. Rescue of *N. crassa aod-5*mutant strain with *A. nidulans aod-5*. Conidia from the *aod-5* mutant strain were transformed by electroporation with 2μg linearized pBNAsp5 plasmid and plated onto medium containing benomyl or antimycin A.

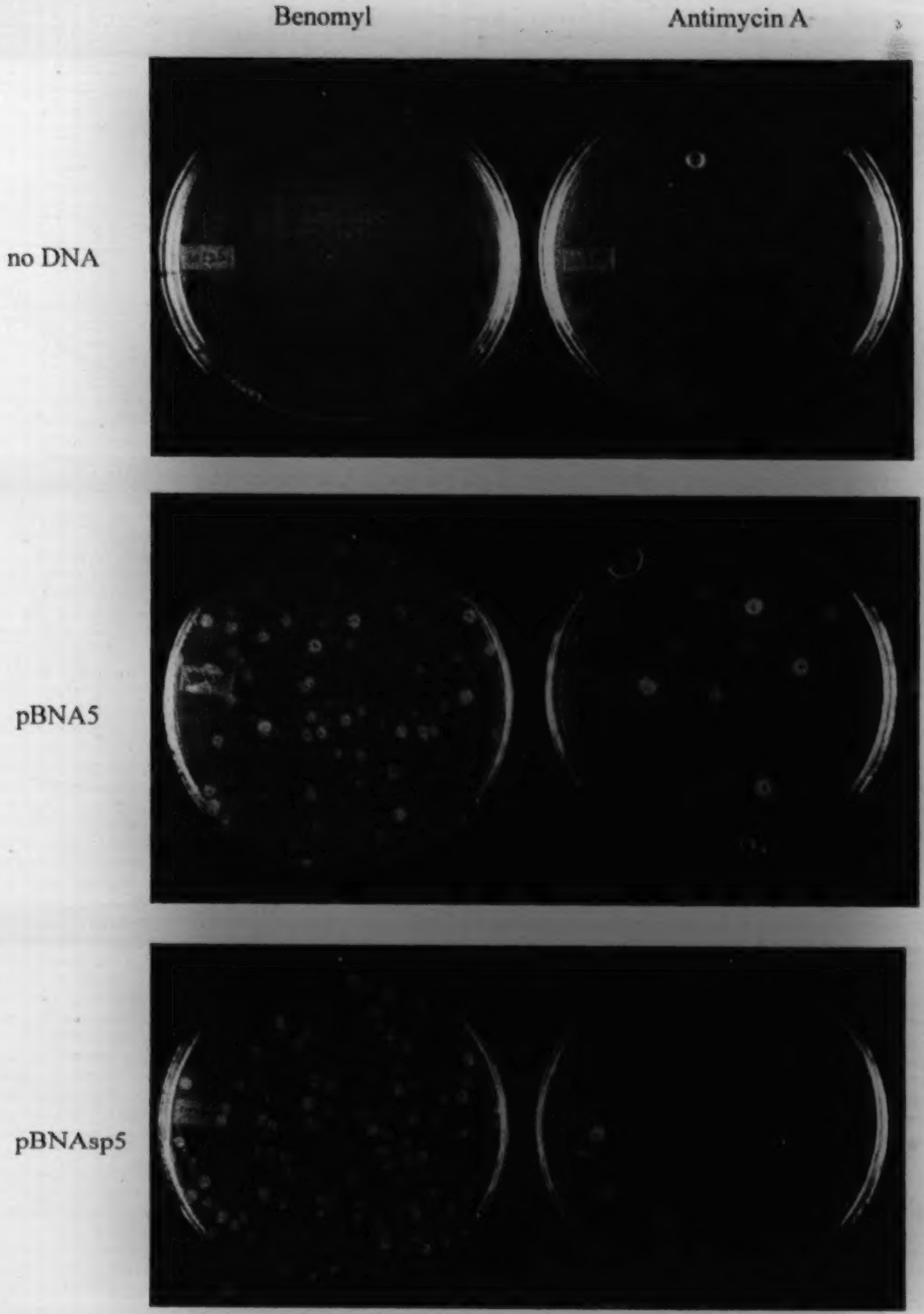
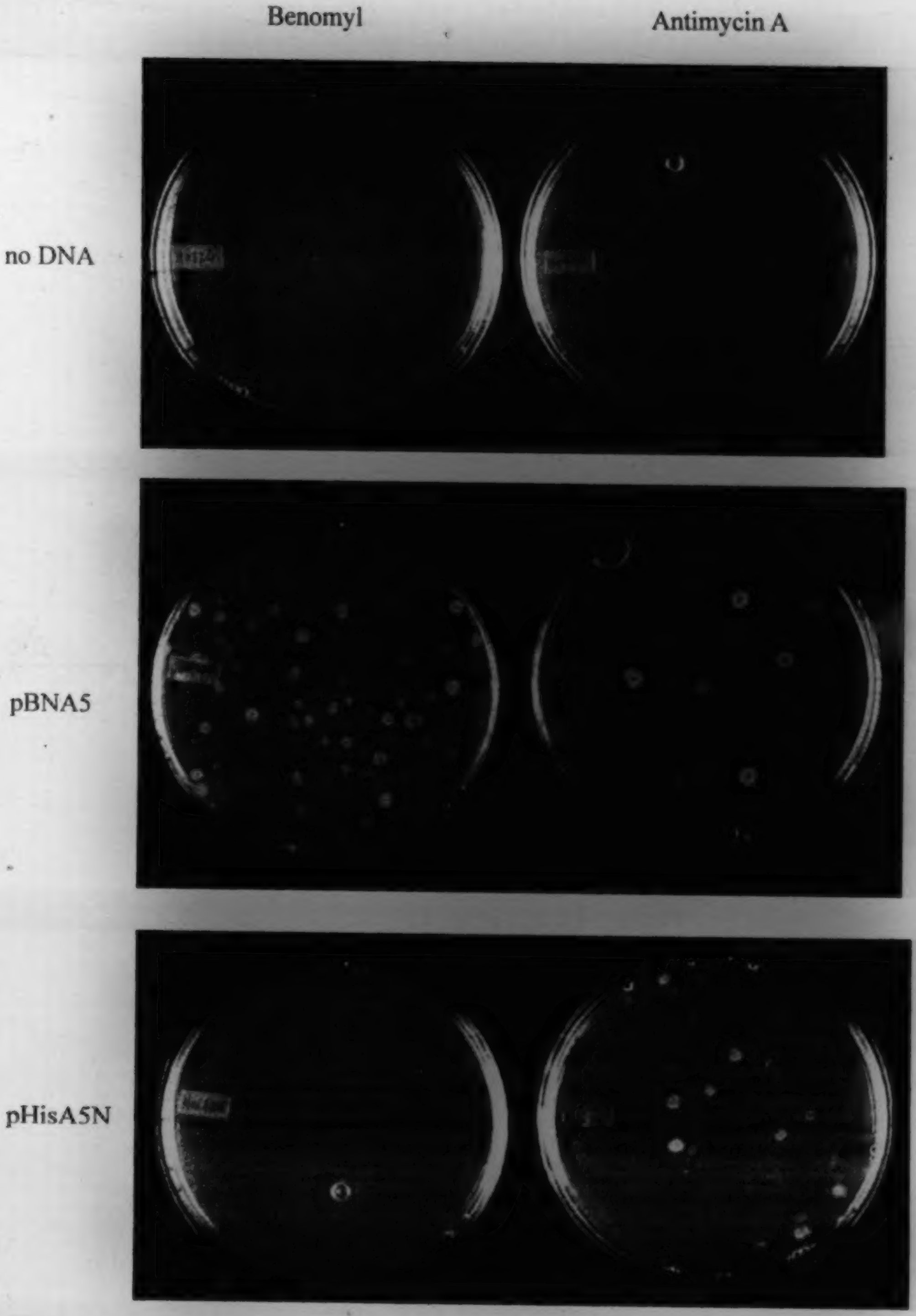
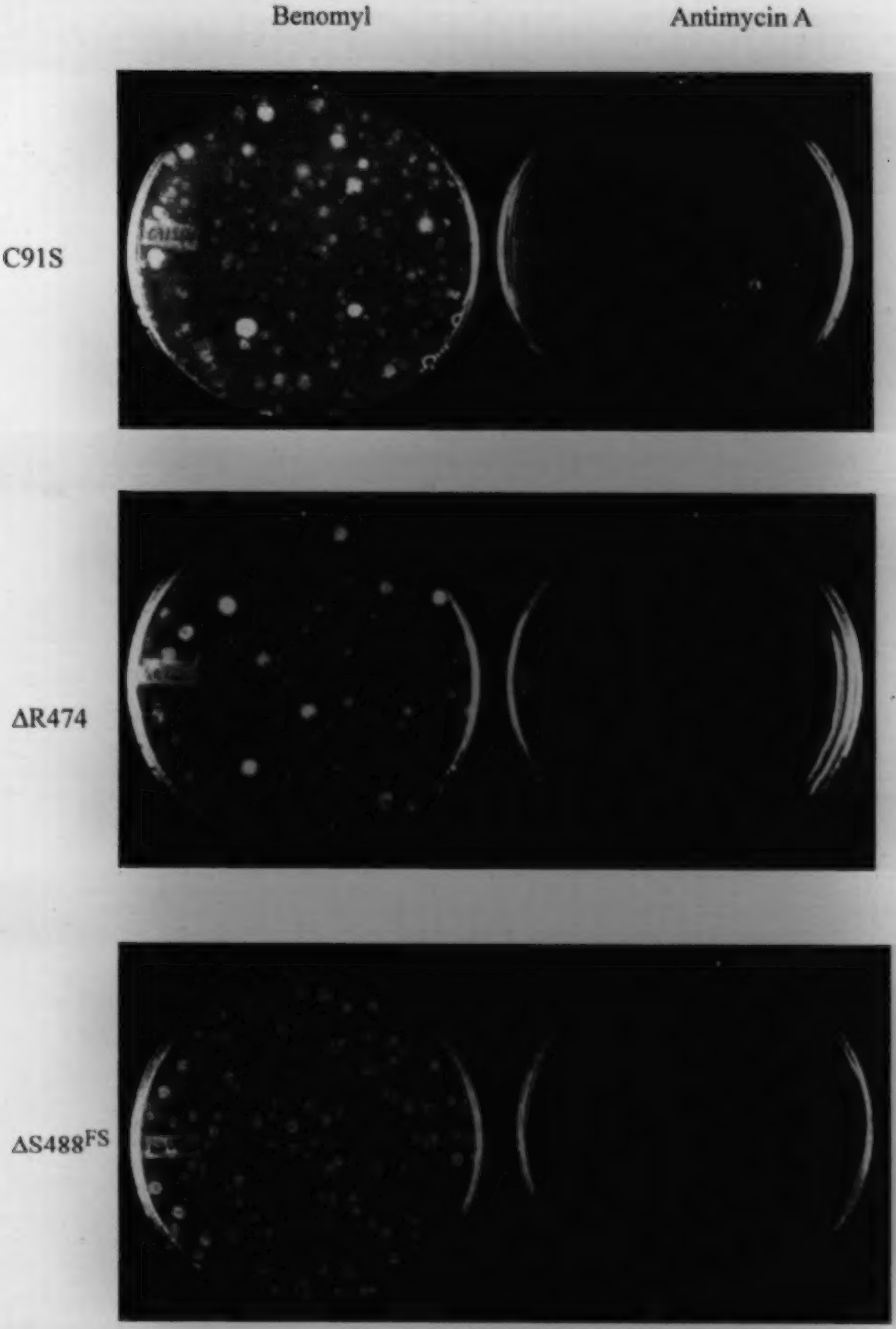

Benomyl
Antimycin A
no DNA
pBNA5
pBNAsp5

Figure 15. Electroporation with plasmids carrying *aod-5* containing site-directed mutantions. Conidia from the *aod-5* mutant strain were transformed by electroporation with 2 μg of the indicated linearized plasmid DNA and plated onto medium containing benomyl or antimycin A. Transformation with plasmids pBNA5 and pHisA5N serve as positive controls. Plasmid pHisA5N contains an *aod-5* gene with a 6-his tag at the N-terminus but does not carry the gene for benomyl resistance. Plasmid pBAN5 contains a wild-type *aod-5* gene and a gene that confers resistance to benomyl.


Benomyl
Antimycin A
no DNA
pBNA5
pHisA5N


Benomyl
Antimycin A
C91S
ΔR474
ΔS488^FS

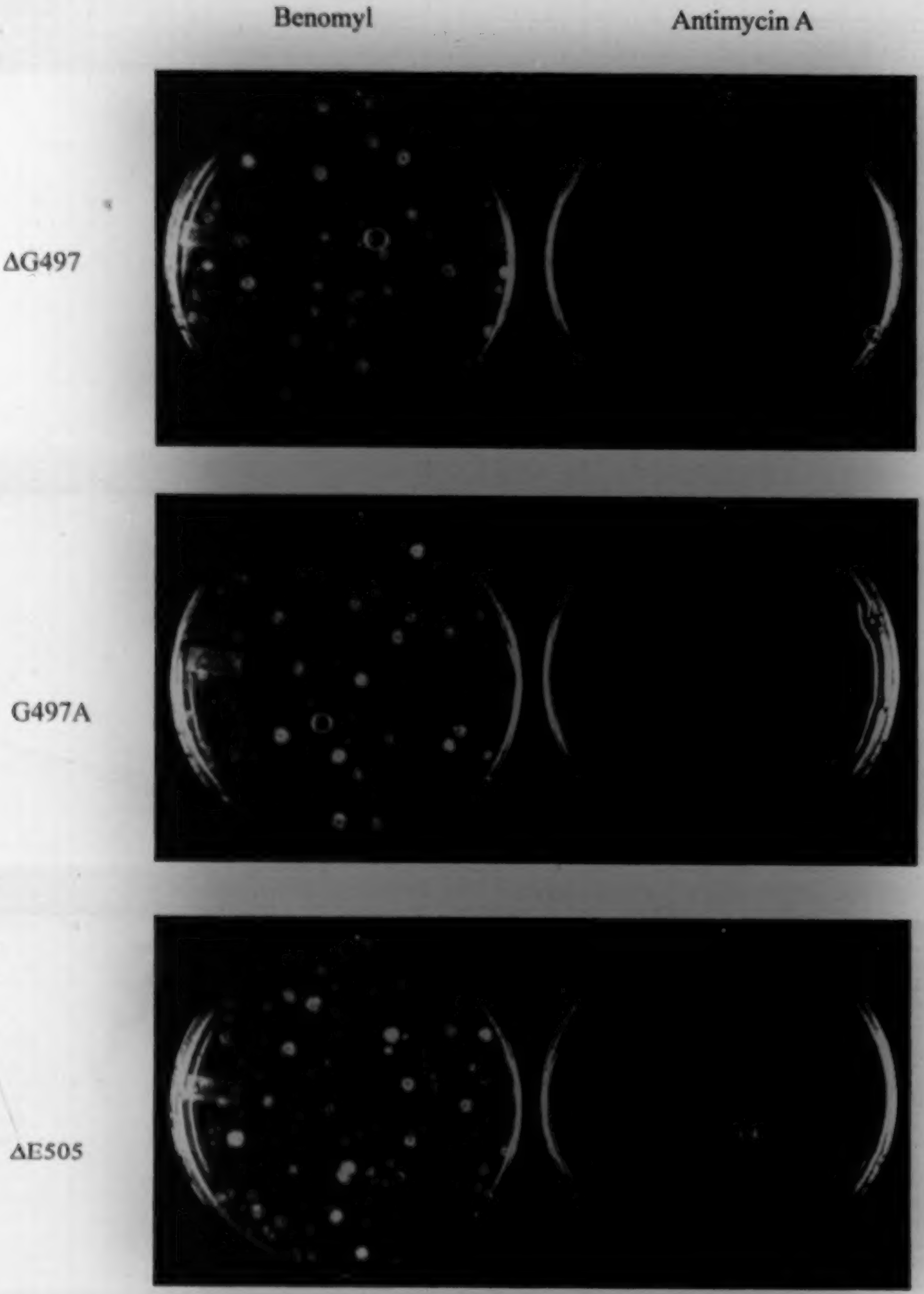
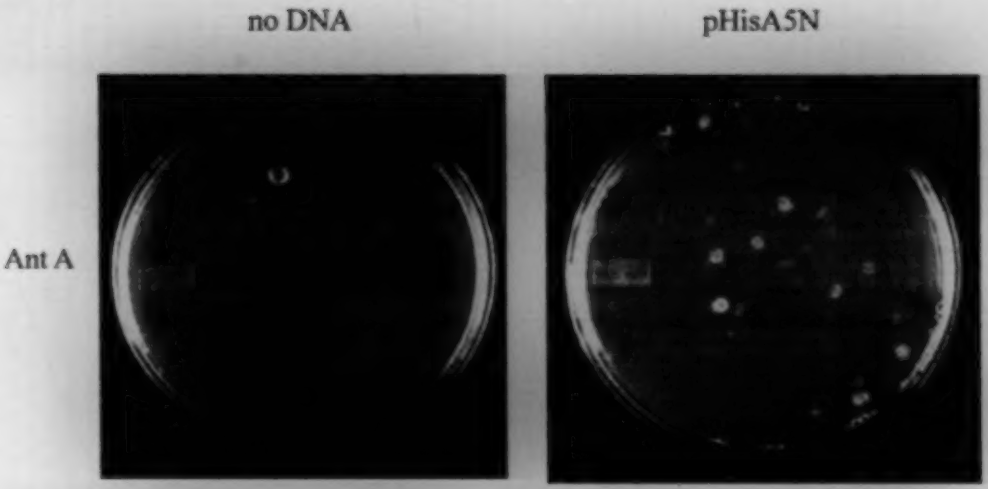

Benomyl
Antimycin A
ΔG497
G497A
ΔE505

Figure 16. Growth of his-tag AOD5 strain. A. Conidia from the *aod-5* mutant strain were transformed by electroporation with 2 μg of pHisA5N linearized plasmid DNA or no DNA and plated onto medium containing antimycin A. B. 10 μl of conidia from a wild-type strain (NCN233), the *aod-5* mutant strain (PL40-23), and the *aod-5* mutant strain transformed with the N-terminal His Tag form of *aod-5* (pHisA5N) were spotted onto non-inducing medium as well as medium containing antimycin A or chloramphenicol; the number of conidia spotted is indicated above each column. Plates were incubated at 30°C for the indicated times.


no DNA
pHisA5N
Ant A

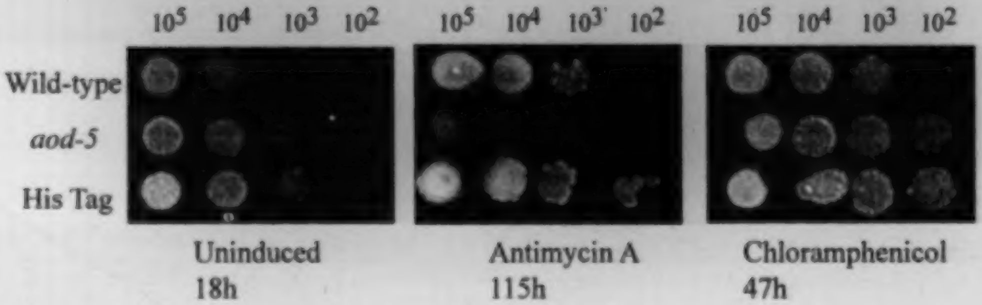

10^5
10^4
10^3
10^2
10^5
10^4
10^3
10^2
10^5
10^4
10^3
10^2
Wild-type
aod-5
His Tag
Uninduced
18h
Antimycin A
115h
Chloramphenicol
47h

Figure 17. Western analysis of nuclear proteins with histone antibody. Nuclei and whole cell extract were isolated from a wild-type strain (NCN233) and the *aod-5* mutant strain (whole cell extract isolated by M. Chae). 50 µg was analyzed by SDS-PAGE, blotted to nitrocellulose and immunodecorated with antiserum against bulk *S. cerevisiae* histone H3 from M. Schultz.

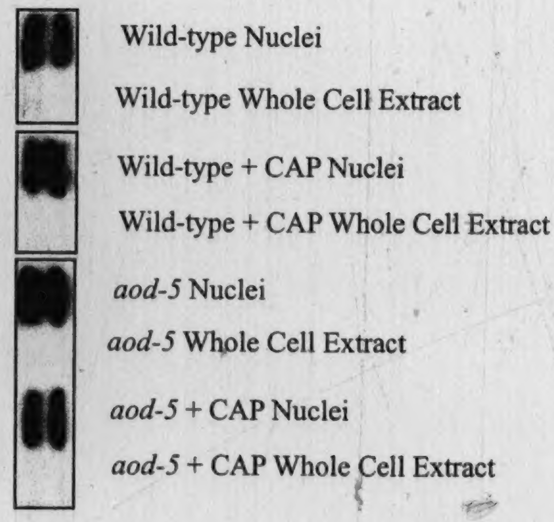

Wild-type Nuclei
Wild-type Whole Cell Extract
Wild-type + CAP Nuclei
Wild-type + CAP Whole Cell Extract
aod-5 Nuclei
aod-5 Whole Cell Extract
aod-5 + CAP Nuclei
aod-5 + CAP Whole Cell Extract

Figure 18. Western analysis of nuclear proteins with penta-his antibody. 25 μg nuclei and 25 μg cytosol were isolated from a wild-type strain (NCN233) and a strain carrying the N-terminal his tag version of AOD5 (HisA5N-1). Protein from each fraction was passed over a NiNTA column and eluted with a Tris-HCl/SDS solution. The purified protein was analyzed by SDS-PAGE, blotted to nitrocellulose and immunodecorated with antiserum against a penta-his sequence.

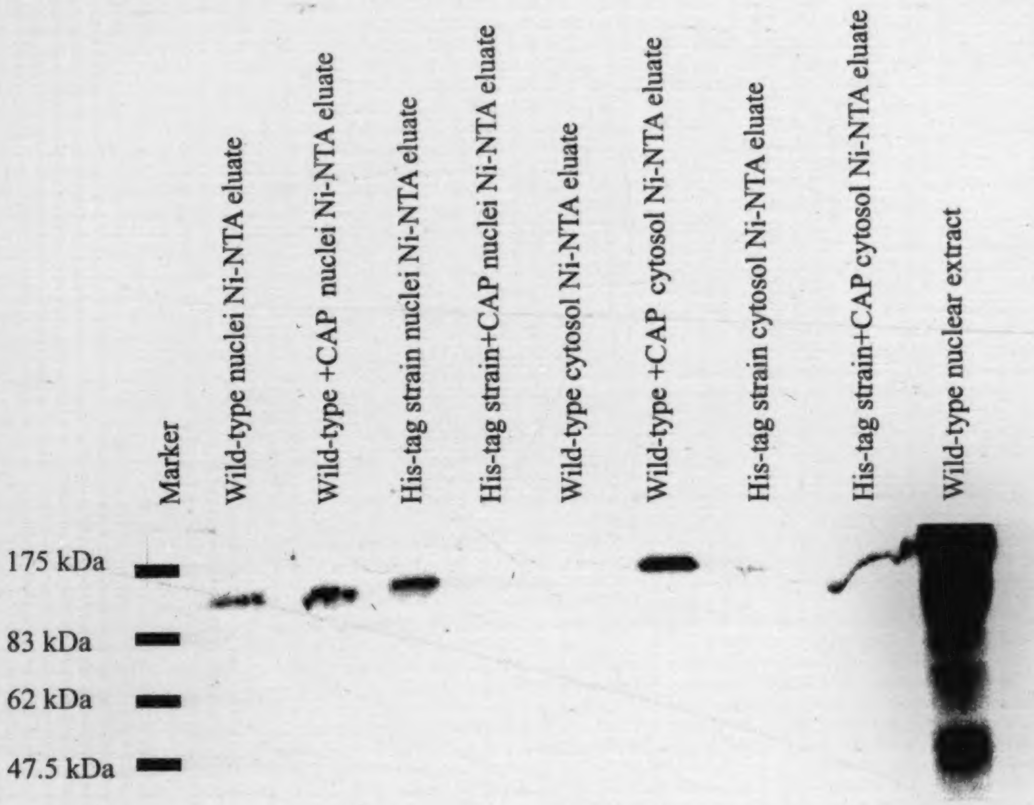

175 kDa
83 kDa
62 kDa
47.5 kDa
Marker
Wild-type nuclei Ni-NTA eluate
Wild-type +CAP nuclei Ni-NTA eluate
His-tag strain nuclei Ni-NTA eluate
His-tag strain+CAP nuclei Ni-NTA eluate
Wild-type cytosol Ni-NTA eluate
Wild-type +CAP cytosol Ni-NTA eluate
His-tag strain cytosol Ni-NTA eluate
His-tag strain+CAP cytosol Ni-NTA eluate
Wild-type nuclear extract

Figure 19. AOD1 cellular localization. Mitochondria and a postmitochondrial supernatant (cytosol) were isolated from wild-type (NCN233) or *aod-6* cultures grown in non-inducing conditions or in the presence of chloramphenicol (CAP). 50 μg of mitochondrial protein and 50 μg of cytosolic protein from each culture were analyzed by SDS-PAGE, blotted to nitrocellulose and immunodecorated with the indicated antiserum to Tom70 (a 70-kDa MOM protein), the AOD1 protein (36 kDa), or yeast actin (a 40 kDa cytosolic protein).

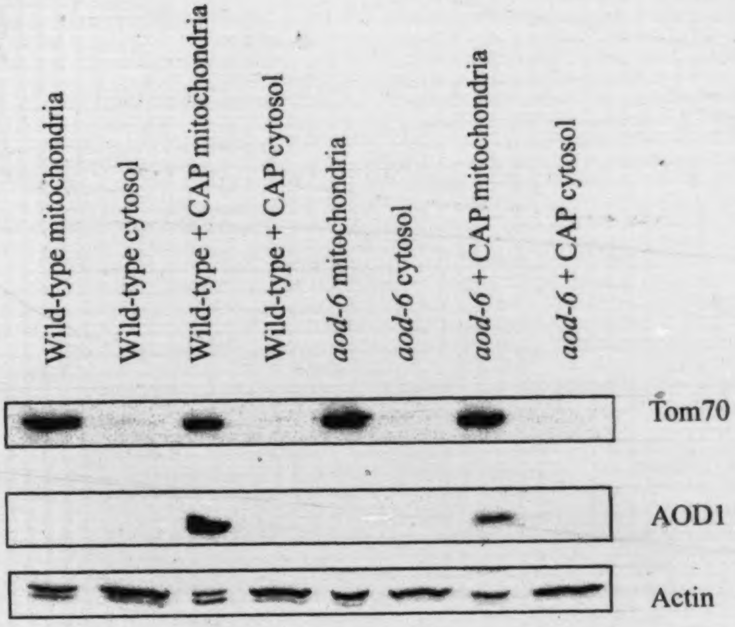

Wild-type mitochondria
Wild-type cytosol
Wild-type + CAP mitochondria
Wild-type + CAP cytosol
aod-6 mitochondria
aod-6 cytosol
aod-6 + CAP mitochondria
aod-6 + CAP cytosol
Tom70
AOD1
Actin

Figure 20. Sonication of mitochondria. Mitochondria were isolated from (A) wild-type (NCNC233) (B) the *aod-6* mutant strain (EL62-2) (C) parent strain of EL62-2 7262 and (D) reporter strain T11-76 cultures grown in the presence of chloramphenicol. 50 µg of mitochondria were sonicated for 1, 2, 4, 6 or 12 bursts of 10 s, followed by centrifugation at 45,000 rpm for 1 h. The samples in the untreated lanes were not sonicated, but were subjected to centrifugation. Soluble proteins in the supernatant were precipitated with TCA. 50 µg of mitochondrial protein (M) as well as the pellet (P) and supernatant (S) of the centrifugation were subjected to SDS-PAGE, blotted to nitrocellulose and immunodecorated with indicated antiserum to Hsp70 (a 70-kDa mitochondrial matrix protein), AOD1 protein (36 kDa), or Tim17 (a 17 kDa MIM protein).

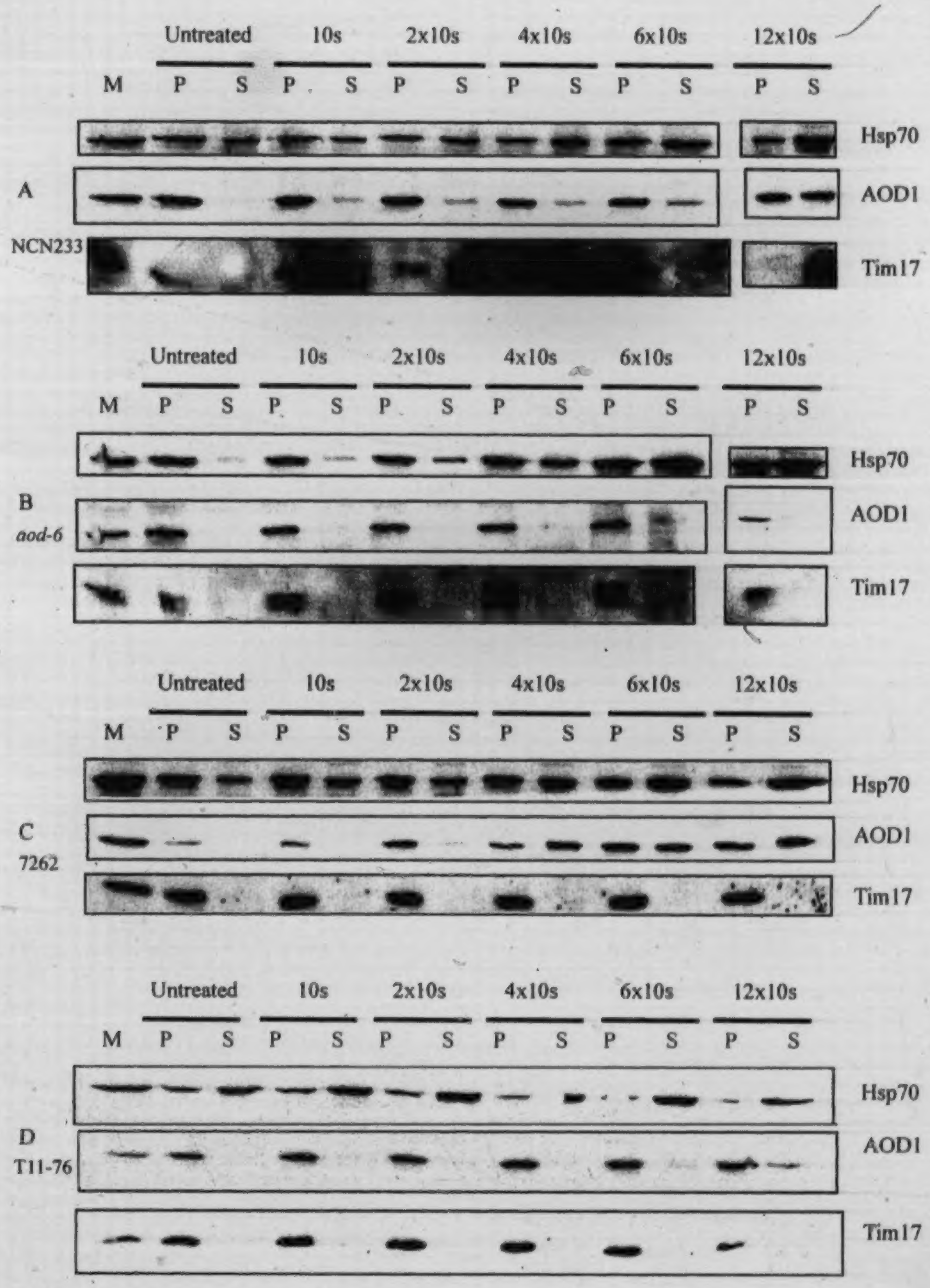
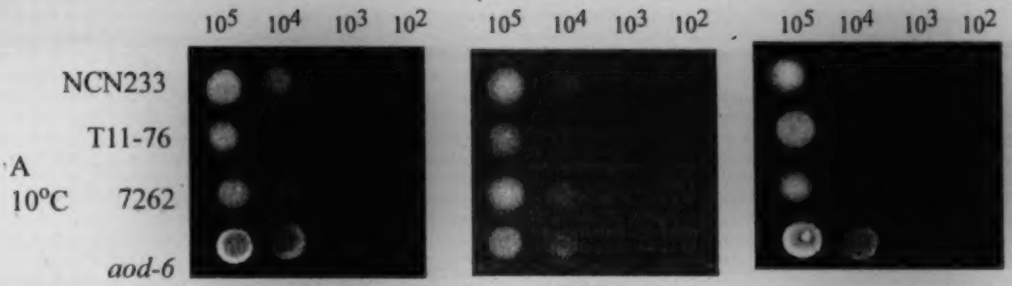

Untreated
10s
2x10s
4x10s
6x10s
12x10s
M
P
S
Hsp70
AOD1
Tim17
A
NCN233
B
aod-6
C
7262
D
T11-76

Figure 21. Growth of *aod-6* at different temperatures. 10 µl of conidia from a wild-type strain (NCN233), the reporter strain (T11-76), a parent of the *aod-6* strain (7262) and the *aod-6* mutant strain (EL62-2), were spotted onto non-inducing medium as well as medium containing antimycin A or chloramphenicol; the number of conidia spotted is indicated above each column. Plates were incubated at 10°C (Panel A), 30°C (Panel B) or 37°C (Panel C) for the indicated times.

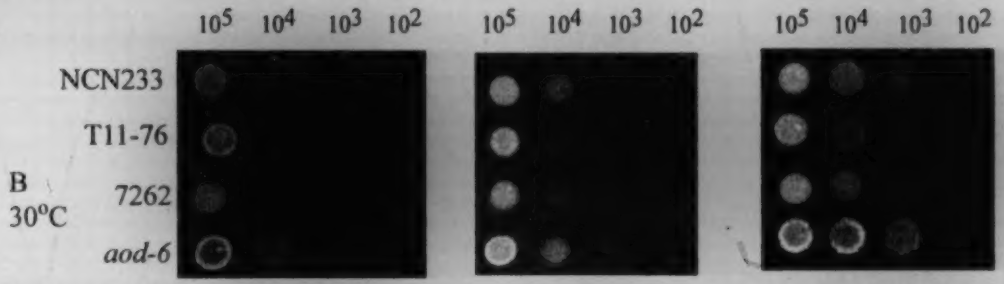


A
10°C
NCN233
T11-76
7262
aod-6
10^5 10^4 10^3 10^2

Uninduced
117h

Antimycin A
140h

Chloramphenicol
117h

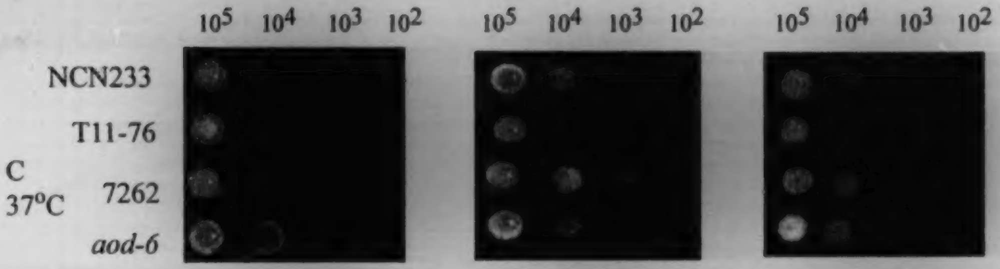


B
30°C
NCN233
T11-76
7262
aod-6
10^5 10^4 10^3 10^2

Uninduced
21h

Antimycin A
44h

Chloramphenicol
44h



C
37°C
NCN233
T11-76
7262
aod-6
10^5 10^4 10^3 10^2

Uninduced
22h

Antimycin A
90h

Chloramphenicol
47h

Figure 22. The oxa-1^{RIP} sheltered heterokaryon. This strain contains two distinct nuclei, as represented by the two circles. Nucleus 1 carries a RIPed copy of *oxa-1*, is auxotrophic for lysine and leucine, and also carries the gene for cycloheximide resistance. Nucleus 2 has a functional copy of *oxa-1*, is auxotrophic for inositol and is sensitive to cycloheximide. Since *oxa-1* is an essential gene in *N. crassa* the mutation must be sheltered by a wild-type copy of the gene. When the heterokaryon is grown in medium containing lysine, leucine and cycloheximide nucleus 1 will become more abundant relative to nucleus 2, and the culture will be OXA1 deficient. A minimal amount of protein necessary for viability will be produced by nucleus 2, which will be related to the concentration of cycloheximide.

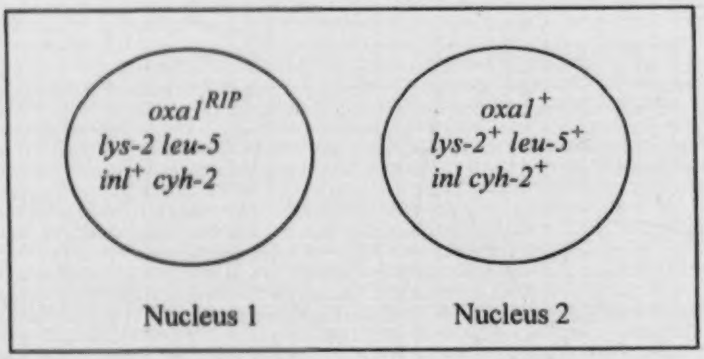

oxa1^RIP
lys-2 leu-5
inl+ cyh-2
oxa1+
lys-2+ leu-5+
inl cyh-2+
Nucleus 1
Nucleus 2

Figure 23. Import of AOD1 into wild-type mitochondria. 50 µg of wild-type (NCN233) mitochondria from cultures grown in the presence of chloramphenicol (CAP) were treated with 0.1 M sodium carbonate (pH 11.5) for 30 min on ice. The membrane fraction was collected by centrifugation at 45,000 rpm for 1 h and soluble proteins in the supernatant were precipitated with TCA. A control sample of 50 µg of mitochondria was not subjected to alkaline extraction and centrifugation. The samples were analyzed by SDS-PAGE, blotted to nitrocellulose and immunodecorated with indicated antiserum to Hsp70 (a 70-kDa mitochondrial matrix protein), AOD1 protein (36 kDa), or Tom22 (a 22 kDa MOM protein).

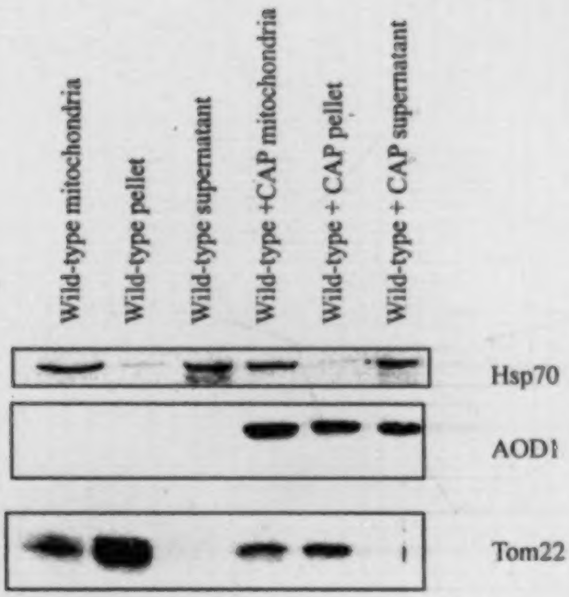

Wild-type mitochondria
Wild-type pellet
Wild-type supernatant
Wild-type +CAP mitochondria
Wild-type + CAP pellet
Wild-type + CAP supernatant
Hsp70
AOD1
Tom22

Figure 24 Alkaline extraction of AOD1 after import. Radiolabelled AOD1 precursor protein was incubated with mitochondria isolated from a wild-type strain (NCN251) for the indicated times. Each incubation time was performed in duplicate. One sample was used directly for SDS-PAGE (M). The second sample was treated with 0.1 M sodium carbonate, (pH 11.5) for 30 min on ice. The membrane fraction (P) was collected by centrifugation at 45,000 rpm for 1 h and soluble proteins (S) in the supernatant were precipitated with TCA. A sample of the radioactive lysate (L) was included for comparison. All samples were subjected to SDS-PAGE and blotted to nitrocellulose. The blot was then exposed to x-ray film.

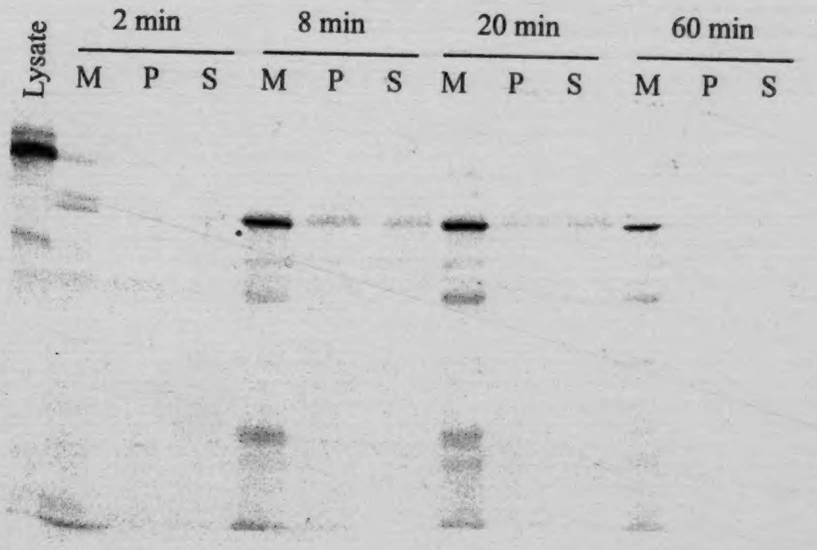
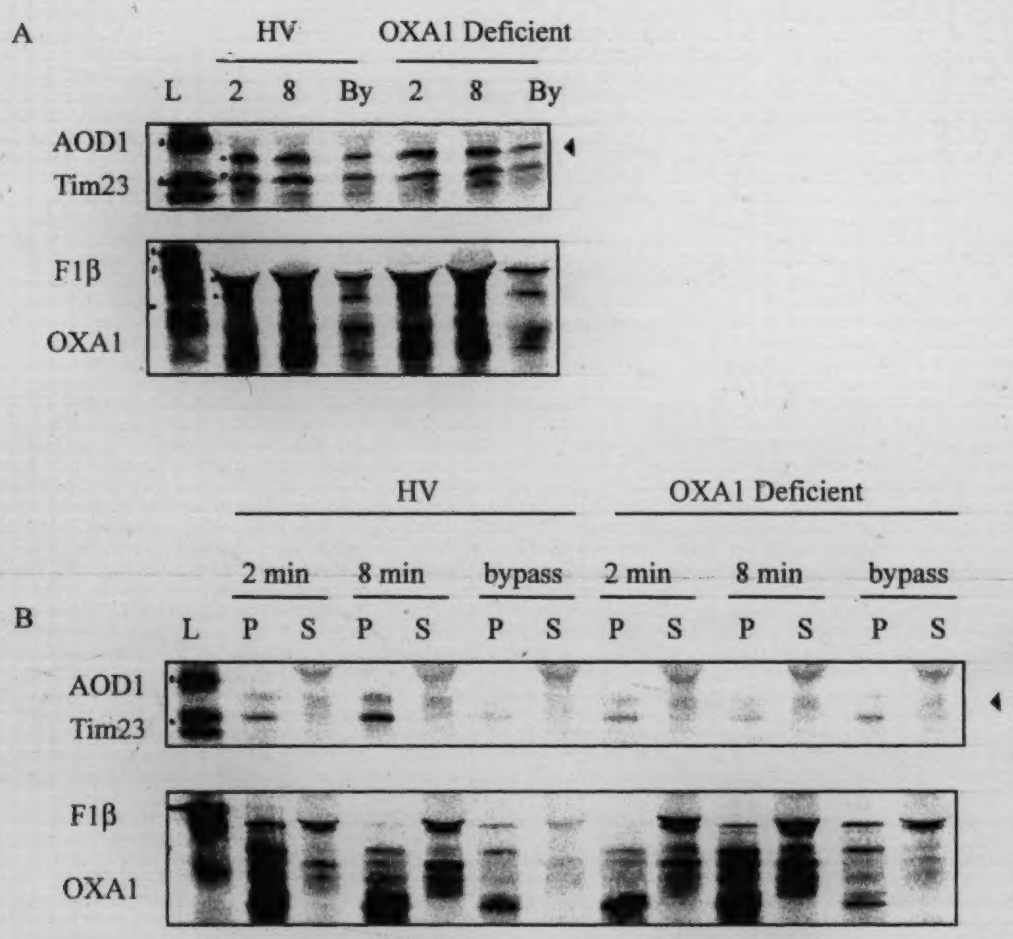

Lysate
2 min
8 min
20 min
60 min
M P S
M P S
M P S
M P S

Figure 25. Import of AOD1 into OXA1-deficient mitochondria. Mitochondria were isolated from a control strain (HV) and the oxa-1^{RIP} strain (ox80-15-1) both grown in the presence of cycloheximide (30 μg/ml final concentration). Radiolabelled precursor proteins of AOD1, OXA1, Tim23 and F1β precursor protein were incubated with these mitochondria at 25°C for 2 or 8 min. One 8 min incubation was performed after trypsin digestion (By or Bypass) which digested the outer receptors of the TOM complex. Each incubation time was performed in duplicate. One sample was used directly for SDS-PAGE (A). The second sample was treated with 0.1 M sodium carbonate, (pH 11.5) for 30 min on ice (B). The membrane fraction (P) was collected by centrifugation at 45,000 rpm for 1 h and soluble proteins (S) in the supernatant were precipitated with TCA. A sample of the radioactive lysate (L) was included for comparison. All samples were subjected to SDS-PAGE and blotted to nitrocellulose. The blot was then exposed to x-ray film. Proteins AOD1, F1β and OXA1 *in vitro* synthesized precursor proteins contain targeting sequences which are cleaved upon import into mitochondria. The precursor proteins in the lysate are therefore larger than the mature proteins found inside the mitochondria after import has taken place. The black arrowhead indicates the position of processed AOD1 protein.


A
HV
OXA1 Deficient
L
2
8
By
2
8
By
AOD1
Tim23
F1β
OXA1
B
HV
OXA1 Deficient
2 min
8 min
bypass
2 min
8 min
bypass
L
P
S
P
S
P
S
P
S
P
S
P
S
AOD1
Tim23
F1β
OXA1

4. Discussion

4.1 An alternative oxidase regulatory pathway

Although alternative oxidase activity has been studied in many plants (JUSZCZUK and RYCHTER 2003) and fungi (JOSEPH-HORNE *et al.* 2001), *N. crassa* remains the only species for which regulatory loci have been identified (BERTRAND *et al.* 1983; DESCHENEAU *et al.* 2005).

An EMS mutagenesis screen of a reporter strain generated fifteen mutants where *aod-1* was not induced when grown in the presence of chloramphenicol, a condition which normally induces the enzyme. One of these mutants did not actually affect *aod-1* directly since it was a chloramphenicol resistance gene *chl-2* (DESCHENEAU *et al.* 2005). Complementation analysis of the remaining putative regulatory mutants identified five novel complementation groups (*aod-4, aod-5, aod-6, aod-7* and *aod*-8) and one allele (E3) of the previously known regulatory locus *aod-2*. Four of the novel complementation groups (*aod-5, aod-6, aod-7* and *aod*-8) are represented by a single member, while one group (*aod-4*) contains seven mutants (E1, E5, E7, E9, E11, E12, E14). The isolation of a number of different mutants shows that alternative oxidase regulation in *N. crassa* is a complex process involving several loci. It is also clear that the mutagenesis screen did not reach saturation, since only one novel complementation group contains more than one mutant. This suggests that additional genes may be involved in alternative oxidase regulation in *N. crassa*.

4.2 At least two transcription factors are involved in alternative oxidase regulation

The *aod-5* mutant was mapped and subsequently cloned. This gene is predicted to encode a transcription factor of the Zn(II)2Cys6 family. This family of transcription factors is specific to fungal species, and is involved in regulating many different processes, including nitrogen utilization (FU *et al.* 1989), amino acid synthesis (FRIDEN *et al.* 1989; MAMANE *et al.* 1998; ZHOU *et al.* 1990), and metabolic regulation in response to different carbon sources such as glucose or galactose (LESAGE *et al.* 1996; ZHANG and GUARENTE 1994). Nearly all members of this family are transcriptional activators (SCHJERLING and HOLMBERG 1996).

The zinc cluster domain is responsible for binding DNA at a pair of trinucleotide repeats in the promoter region of target genes. The transcription factors bind these repeats as a homodimer or heterodimer, with the zinc cluster domain from each protein binding one of the repeats (TODD and ANDRIANOPOULOS 1997). Recently, the previously known regulatory locus *aod-2* was cloned by Cheryl Nargang, a technician in the lab. The protein encoded by *aod-2* is also predicted to be a Zn(II)2Cys6 binuclear cluster protein like *aod-5*. Interestingly, there is a CGG trinucleotide repeat motif in the upstream region of *aod-1*. The motif is found in a region identified as necessary for *aod-1* induction (M. Chae personal communication). It is possible that an AOD2/AOD5 heterodimer binds the upstream region of *aod-1* to induce transcription. The lab is currently studying potential binding activities at this motif.

AOD5 is also predicted to contain a PAS domain. This conserved protein fold is found in proteins from bacteria to higher eukaryotes. This domain has several functions, including interacting with other PAS domains and binding various cofactors. The main function of the PAS domain is signal transduction. Some PAS domain containing proteins are able to sense changes in redox potential or respond to changes in light (TAYLOR and ZHULIN 1999). The fact that AOD5 contains a PAS domain is also consistent with its potential role in regulating alternative oxidase production. *aod-1* is specifically expressed when electron flow through the ETC is disrupted. It is possible that AOD5 is activated by signals transduced through the PAS domain, *via* a bound heme group or other cofactor that might be sensitive to cellular redox changes. The activated protein could then bind to its particular target and activate gene expression.

4.2.1 Mutations in Conserved Amino Acids Eliminate AOD5 Function

Several conserved amino acids in the predicted motifs of AOD5 were deleted or replaced to confirm the importance of these residues and motifs. One cysteine in the zinc cluster was replaced with a serine, while several residues in and near the PAS domain were deleted or altered. None of these mutations was able to rescue the *aod-5* mutant strain. This confirms the importance of these domains. Interestingly, mutant E3, an allele of *aod-2* that was identified in this study, contains a mutation that alters one of the cysteines in the zinc cluster domain of AOD2 (F. Nargang, personal communication). This confirms that changing one cysteine residue is sufficient to eliminate function of AOD2 and of AOD5

4.2.2 Expression of *aod-5*

aod-5 appears to be expressed at a very low level as even when large quantities of RNA were used in a Northern blot, no signal could be detected. Other *N. crassa* genes in this transcription factor family are also expressed at extremely low levels (FU *et al.* 1989). RT-PCR showed that the transcript is present constitutively in wild-type and the *aod-5* mutant cells. This suggests that *aod-5* is not regulated at the level of transcription, though I cannot formally rule out the possibility that transcript levels change under conditions where *aod-1* is induced. Many previously studied zinc cluster proteins are also expressed constitutively and are activated by post-translational modifications in response to external stimuli (HE *et al.* 2005; LESAGE *et al.* 1996; SCHWERDTFEGER and LINDEN 2000).

4.2.3 AOD5 Protein

Attempts were made to detect AOD5 protein and to characterize its localization. Two AOD5:DHFR fusion proteins were used in an attempt to generate antibodies in guinea pigs and rabbits. In both cases the antiserum reacted strongly with the fusion protein, but also reacted with purified DHFR, a high molecular weight protein in the fusion protein NiNTA eluate, and a variety of other proteins in *N. crassa* nuclear extract. The antisera failed to react with AOD5 full length protein purified from *E. coli*. Both C- and N-terminal his tag versions of AOD5 were created. Both tagged forms of the protein were able to rescue the *aod-5* mutation (Figure 15), and were therefore functional in *N. crassa*. The protein was not, however, detected in nuclear extract or cytosol that had been purified by NiNTA chromatography (Figure 18). This suggested that the AOD5 protein is expressed at very low levels in the cell or that the his tag is not accessible to the penta-his antibody in this construct. As a transcription factor, very little protein may be needed for its cellular function.

4.3 Other EMS Mutants

While the identity of the other mutants isolated in the screen is as yet unknown, one can speculate on some possible roles. Some zinc cluster proteins are known to be regulated by phosphorylation (CHARBON *et al.* 2004; HE *et al.* 2005; LESAGE *et al.* 1996; LIAO and BUTOW 1993; ROTHERMEL *et al.* 1997; SCHAFMEIER *et al.* 2005; SEKITO *et al.* 2000) so it is conceivable that the activation of AOD5 or AOD2 is regulated by a phosphorylation event. One of the other EMS mutants could be a kinase or phosphatase necessary for that regulation. The identification of two transcription factors does not complete the *aod-1* induction signaling pathway. Other mutants from the screen could encode proteins involved in signal transmission from mitochondria to the nucleus. They could be transporters in the mitochondria membranes that carry signaling molecules out of mitochondria into the cytosol or the nucleus. It is possible that the translocation of AOD2 and AOD5 to the nucleus is a component of their regulation. Other mutants could affect factors required for this translocation, such as chaperones or shuttle proteins. Since there are several other loci involved in *aod-1* regulation, the pathway must have a number of components.

4.4 AOD 1 Assembly

Little is known about how AOD1 is assembled into its functional conformation at the MIM. *In vitro* import assays followed by carbonate (pH11.5) extractions showed that AOD1 newly imported into wild-type mitochondria was found in equal amounts in the membranous pellet and supernatant (Figure 24). This ratio of protein between pellet and supernatant was also seen when mitochondria treated with sodium carbonate (pH11.5) were analyzed by Western blot (Figure 23).

The OXA1 (NARGANG *et al.* 2002) and OXA2 proteins (FUNES *et al.* 2004) are required for assembly of ETC components into the MIM from the matrix. Since alternative oxidase is thought to be an interfacial membrane protein and interact with one lipid bilayer of the MIM, it seemed possible that OXA1 may be required for proper alternative oxidase assembly. Alternative oxidase assembly is not impaired in *oxa2 N. crassa* mutants (FUNES *et al.* 2004) where mitochondrial AOD1 levels were not decreased, even when other ETC components did show decreased assembly into the MIM. AOD1 imported into oxa-1^{RIP} mitochondria and subjected to carbonate (pH 11.5) extraction showed the protein to be imported at the same level as in control mitochondria and present in roughly equal amounts in the pellet and supernatant (Figure 25), as was seen for wild-type mitochondria (Figure 24). In contrast, less OXA1 protein was imported into mitochondria from the OXA1-deficient strain. These data suggest that OXA1 is not required for AOD1 assembly at the MIM.

Sonication experiments showed that while a portion of the matrix protein Hsp70 was easily released by short sonication treatments, AOD1 remained associated with the membrane after repeated sonication bursts (Figure 20). Together these data provide support that AOD1 in *N. crassa* mitochondria is associated with the MIM and only a fraction is removed by carbonate treatment (Figures 23 and 24). AOD1 does not exist in a pool of soluble protein in the matrix with only a small portion associated with the MIM.

4.5 The *aod-6* Mutant Strain

The phenotype of the *aod-6* mutant is complex and difficult to explain. This strain is able to grow slowly in the presence of antimycin A, even though it was isolated by the filtration enrichment mutagenesis method that was designed to eliminate cells able to grow in the presence of antimycin A. The *aod-6* mutant accumulates *aod-1* mRNA and has AOD1 protein in its mitochondria when grown under inducing conditions, but has virtually no cyanide insensitive respiration. These observations suggest a defect in the function of the AOD1 protein, but the endogenous *aod-*1 gene does not have any mutations. In addition, in the *aod-6* strain the tyrosinase reporter is not activated when grown in the presence of chloramphenicol. The lack of reporter activation suggests the presence of an *aod-1* regulatory defect. Sequence analysis identified one mutation in the reporter promoter but at a position that is unlikely to affect its function. The simplest explanation for the entire *aod-6* phenotype is that one mutation affects the accumulation and function of the AOD1 protein, while a second mutation affects reporter function in this strain.

Since the *aod-6* strain is able to grow in the presence of antimycin A, albeit more slowly, it would be difficult to rescue this mutation by sib selection. Since it was plausible that the mutant strain might suffer from an error in correct assembly of AOD1 at the MIM, experiments were done to try to characterize the mutation from a functional perspective.

4.5.1 AOD1 Localization and Assembly in *aod-6*.

A defect in AOD1 localization within the cell could explain the reduced levels of AOD1 found in *aod-6* mitochondria. To examine this possibility, mitochondria and a post-mitochondrial supernatant were isolated from wild-type and *aod-6* strains. In both cases no AOD1 protein was found in the cytosol while a large amount of protein was found in the mitochondrial fraction. The *aod-6* strain does not, therefore, suffer from a defect in AOD1 protein targeting.

While apparently correctly targeted to mitochondria, AOD1 may not be localizing to the proper mitochondrial compartment. A way to assess AOD1 association with the MIM is by sonication of mitochondria. In control mitochondria of strains NCN233 and 7262, AOD1 is increasingly solubilized by increased sonication (Figure 20), reaching approximately apportionment between pellet and supernatant at the longest sonication times tested. In contrast, AOD1 from the *aod-6* mutant and from T11-76 mitochondria is only found in the supernatant after lengthy sonication. AOD1 protein appears to be more tightly associated with the membrane in the mutant strain and one of its parents than in other control strains. The nature of the change and that factor causing it are not clear.

4.6 The *aod-8* Mutant Strain

Like the *aod-6* mutant strain, the *aod-8* mutant fails to activate the reporter (Figure 2), has little cyanide insensitive respiration when grown under inducing conditions (Figure 3), but is able to grow in the presence of antimycin A (Figure 5) and accumulate AOD1 protein (Figure 7). The sequence of the reporter has been obtained and one mutation of unknown effect found in the promoter region 624 bp upstream of the

ATG. The sequence of the endogenous *aod-1* gene in this strain matches wild-type sequence. Complementation tests show that this mutation is different from the *aod-6* mutation, although there are similarities in phenotype between the two mutants. Like the *aod-6* mutant, this strain is able to grow in the presence of antimycin A, making the identification of the mutation by rescue a more difficult proposition.

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